TECHNICKÁ UNIVERZITA V LIBERCI

FAKULTA TEXTILNÍ

DISERTAČNÍ PRÁCE

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Identification of risk concentrations of hazardous compounds on textiles

Liberec **2014**

Název disertační práce:	Stanovení nebezpečných koncentrací rizikových látek v textiliích Identification of risk concentrations of hazardous compounds on textiles
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Obor doktorského studia:	Textile Material Engineering
Forma studia:	Full-time
Školící pracoviště:	Department of Material Engineering
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Počet stránek textu:	140
Počet obrázků:	105
Počet tabulek:	33

Prohlášení:

Prohlašuji, že předkládanou disertační práci jsem vypracoval samostatně pod vedením školitele Prof. Ing. Jiřiho Militkého, CSc. a s použitím uvedené literatury.

Declaration:

The contents of the thesis are experimental results obtained by the author on the basis of literature and under the supervision of Prof. Ing. Jiří Militký, CSc.

Dedicated to my parents

Anotace

Bavlna je nejdůležitějším textilním celulózovým vláknem na světě používaném k výrobě oděvů a bytových a průmyslových výrobků. Bavlna vždy byla velkou součástí textilního průmyslu a dnes se svými 38% světové textilní spotřeby zaujímá druhé místo hned za polyesterem, který se dostal do popředí. Vyrábí se široké spektrum oděvů z bavlny, jako košile, šaty, dětské oblečení, halenky, obleky, saka, sukně, kalhoty, svetry, šály, punčochové zboží a oblečení pro aktivní spotřebitele, pro svůj měkký omak, dobrou savost, stálobarevnost, vysokou pevnost, snadné šití a manipulaci, lze prát v pračce i chemicky čistit.

Za poslední desítky let rezidua pesticidů v potravinách a plodinách vyvolávají veřejné znepokojení. Mezi mnoha spotřebiteli panuje představa, že ekologicky pěstovaná bavlna je v některých ohledech lepší než bavlna pěstovaná v konvenčním zemědělství. „Organické oblečení“ a „Vyrobeno ekologicky“ jsou často užívanými marketingovými koncepty v dnešní době. Zejména stran znepokojení ohledně pesticidů se trh snaží uspokojit poptávku spotřebitelů, kteří jsou ochotni si za bezpečnost svého zdraví připlatit. Nicméně, stále není jisté, který z výrobních postupů je lepší, co se zbytků pesticidů týká. Zjednodušené přístupy jako organický – dobrý a syntetický – špatný jsou použitelné v reklamě, ale obtížně se zdůvodňují vzhledem k závislosti na mnoha faktorech.

Vzhledem ke svým vlastnostem jsou pesticidy velmi toxické, ale úroveň rizika pro spotřebitele závisí na úrovni vystavení se pesticidům. Pokud zbytky pesticidů nezůstávají v bavlně, neexistuje tím ani žádné riziko pro spotřebitele. Na druhou stranu, pokud používání pesticidů povede k vysokým zbytkům, následkem bude i vyšší riziko.

Tato disertační práce posuzuje míru rizika založeného na procesech s účelem rozhodnout, zda je riziko nízké a přijatelné ze zdravotního hlediska. Není možné stanovit a vyčíslit všechny zbytky pesticidů všech typů bavlny v rámci dostupných zdrojů. Takže pro porovnání vzorků z obou zemědělských oblastí byla zvolena analýza jejich toxických účinků. V úvahu byly vzaty všechny důležité faktory, jako je výběr řádných vzorků, manipulace, předprava (kryogenní homogenizace), extrakce a analýza.

Tato práce je kombinací studia výsledků kvalitativních a kvantitativních analytických měření. Pro kvalitativní analýzu byla použita metoda přístupu biosenzorů a také interakce se zelenými řasami. To bylo zkoumáno měřením bioelektrických signálů způsobených inhibicí enzymatické acetyl-cholinesterázy (AChE) s použitím Analyzátoru biosensorové toxicity (BTA) a Mini Termostatu (MT-1) a sledováním změn signálů způsobených interakcí biologických látek a reziduí. Všechny veličiny podílející se na aktivitě AChE inhibici byly studovány a optimalizovány, jako enzymy a koncentrace substrátu, pufr, pH a doba inkubace. Metoda se používá na vzorky pravé bavlny extrahované různými rozpouštědly. Nejenže jsme schopni odhadnout % inhibice

každého jednotlivého vzorku, ale také můžeme porovnávat tuto inhibici se standardními kontrolními body. Zvláštností této metody je, že všechny vzorky spolu s kontrolními body mohou být testovány v jednom běhu. Celkový čas jednoho kompletního testu byl přibližně 50 - 55 minut. Je to metoda, která nám nabízí snadný způsob, jak zjistit přítomnost organofosforových a karbamátových pesticidů.

Byla provedena další metoda založená na biotestu k identifikaci rizik. Tato metoda studuje interakce zbytkových analytů a zelených řas pro stanovení působících predátorů, kteří ovlivňují jejich běžný životní cyklus měřením inhibice kyslíku vznikajícím fotosyntézou. V této studii jsme viděli změnu chování extraktů z bavlněných vzorků z různých regionů, jež souvisela s variací druhů řas a jejich reakci na toxické látky.

Pro kvantitativní analýzu byla vyvinuta multireziduální metoda pro rozbor 76 pesticidů různých fyzikálně-chemických vlastností. Rezidua pesticidů byla stanovena pomocí plynové chromatografie ve spojení s až trojnásobkem kvadrupólové tandemové hmotnostní spektrometrie (GC-MS/MS). Vyvinutou metodou bylo úspěšně detekováno 57 pesticidů z celkových 76. Kvantifikace a potvrzení pesticidů bylo provedeno v režimu sledování vybrané reakce (SRM). Správnost, opakovatelnost, specifičnost, mez detekce (LOD), limit kvantifikace (LOQ) a aplikovatelnost byly experimentálně stanoveny pro každý reprezentativní analyt. Tato metoda je schopná odhalit pesticidy v reálných vzorcích bavlny. Metoda GC-MS/MS popsaná v této práci poskytuje spolehlivý postup pro stanovení zbytkových pesticidů na bavlněných vláknech. Ukazuje se býti účinnou, rychlou, citlivou a použitelnou pro širokou škálu pesticidů. Zároveň byla splněna všechna validační kritéria dle dokumentu Evropské komise SANCO/12495/2011 pro "Metoda validace a procedury řízení jakosti pro analýzu reziduí pesticidů v potravinách a krmivech". Metoda přinesla dostatečné analytické parametry provedení pro většinu cílových pesticidů a analýza reálných vzorků prokázala její využitelnost pro daný účel.

Annotation

Cotton has been part of the fabric of human existence for thousands of years. Cotton is the most important natural textile fibre, as well as cellulosic textile fiber, in the world, used to produce apparel, home furnishings, and industrial products. Cotton has always been a major part of the textile industry and today provides almost 38% of the world textile consumption, second only to polyester, which recently took the lead. There has been a wide range of cotton made wearing apparel like shirts, dresses, children's wear, active wear, blouses, suits, jackets, skirts, pants, sweaters, hosiery, neckwear due to its unique characteristics of comfortable Soft hand, good absorbency, color retention, machine-washable, dry-cleanable, good strength, easy to handle and sew.

Public concern over pesticide residues in food and crops has been increased for the past several decades. There is a perception among many consumers that organically grown cotton is superior in some aspects to cotton grown with conventional agriculture. 'Organic apparel' and 'organically produced' are now useful marketing concepts. The market will supply the wants of those consumers especially concerned about the safety of pesticide residues and who are willing to pay a premium for reassurance of their health. However, there is still no convincing proof to believe that which production method is better regarding residual pesticides due to the involvement of a lot of factors. A simplistic approach, such as an association of 'natural' with 'good' and 'synthetic' with 'bad' is useful in advertising but is difficult to justify due to the dependency of a lot of factors. A pesticide chemical may be very toxic which can be considered as being dependent on its intrinsic properties but the level of risk to the consumer associated with the chemical will be dependent on the level of exposure. If the pesticide leaves no residues on the cotton, then there would be no risk to the consumer. If on the other hand, the use of the pesticides leads to high residues, then this would result in a risk.

The dissertation is a study of risk assessment based on processes in order to decide if the risk is low and acceptable in scientific terms. It is not possible to identify and quantify all residues of these pesticides on all the types of cotton within available resources. So a comparison of selected cotton samples of both modes of agriculture from the field has been analyzed in terms of their toxic effects. All the important factors for analytical process like proper sampling, handling, pre-treatment (cryogenic homogenization), extraction and analysis have been taken into account.

The thesis is a combination of study of qualitative and quantitative analytical measurements. For qualitative analysis, Biosensor approach and Interaction with algae have been implemented. Measurements of bio-electrical signals caused by enzymatic inhibition of acetyl cholinesterase (AChE) with the use of Biosensor Toxicity Analyzer (BTA) and Mini Thermostat (MT-1) have been studied and the monitoring of changes in signals caused by the interaction of biological substances and residues were evaluated. All the variables involved in AChE inhibition activity have been studied and optimized such as enzyme & substrate concentrations, buffer, pH and incubation time.

The method is utilized for real cotton samples extracted with different solvents. We are not only able to estimate the inhibition % of each individual sample but also we can compare this inhibition with the standard control points. The speciality of this method is that all the samples along with the control points can be tested in one run, The total time utilized for one complete test was approximately 50 ~ 55 minutes. It is a method that offers to different investigators an easy way to detect the presence of organophosphorous and carbamate pesticides.

Another method based on the bioassay for hazard identification has been implemented. The interaction of residual analytes and the green algae has been studied for the determination of intervening predators affecting their normal life cycle by measuring the photosynthetic inhibition of oxygen. In this study a variation in the behaviour of extracts from the cotton samples of different regions has been observed which was related to the variation of algal species in their response to toxic chemicals.

A multiresidue method for analysis of 76 pesticides with different physicochemical properties was developed for quantitative analysis. The pesticide residues were determined by Gas Chromatography coupled to triple Quadrupole Tandem mass spectrometry (GC-MS/MS). 57 out of 76 pesticides were detected successfully by the method developed. Confirmation of pesticide and quantitation was performed in selected-reaction monitoring mode (SRM). Trueness, Repeatability, Specificity, Limit of detection (LOD), Limit of determination (LOQ) and Applicability have been experimentally determined for each individual representative analyte. The method was capable of detecting pesticides in real cotton samples. The GC-MS/MS method described in this work provides a reliable procedure for the determination of residual pesticides on cotton fibers. The procedure was proven to be effective, fast, sensitive and applicable to a wide range of pesticides. All validation criteria mentioned by European Commission document SANCO/12495/2011 for ‘Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed’ were fulfilled. The method gave satisfactory analytical performance parameters for the most of the targeted pesticides and analysis of real samples proved its feasibility for the intended purpose.

Acknowledgement

First of all I must acknowledge the benevolence of Almighty Allah to provide this opportunity and in spite of all my mistakes helping me through invisible sources to accomplish this task.

I would like to express my sincerest appreciation to my supervisor Prof. Jiří Militký who was most dedicated in supporting this work with his precious ideas and helpful discussions. He has been a source of inspiration for me throughout this journey. I would also like to appreciate the organization of Technical University of Liberec for providing the funds to attend several national and international conferences during my studies.

I would like to thank Dr. Jan Krejčí, CEO of BVT Technologies for all the support, assistance and developing the ideas for the method development with Biosensors. Thanks not only for all the scientific input but also for the wonderful time working in a creative and hearty atmosphere. Very much I appreciated the encouragement and support in cooperating with the BVT Technologies.

I would like to dearly thank all members of the department of Mechatronics for giving me not only a friendly and lovely working atmosphere but also having valuable and informative discussions. Thanks Miss Martina Homolková, Miss Eva Kakosová and Mr. Veetek for your cooperation. Special gratitude goes to Mr. Pavel Hrabák for giving me the possibility to perform the testing in their laboratory. He helped me a lot for conducting gas chromatography experiments. His excellent collaboration, inspiring ideas and his willingness to help me with any questions I had, made it very easy for me to make a line of action. The experimental results presented in this thesis would not have been put into reality without his collaboration.

I wish to thank Jiří Chvojka and Martin Stuchlík for their help during several cotton sample preparations. A special thanks also to Prof. Sayed Ibrahim, Prof. Stibor, Prof. Josef Sedlbauer, Dr. Rajesh Mishra and Dr. Mohammad for their guidance and advice throughout my research and help in analysis of the results. I would further like to thank Miss Gabriela, Miss Bohumila Keilova and Miss Monika Mošničková for their support regarding the official matters.

I would like to thank Kateřina Semeráková for her administrative work and official correspondance and the amiable talks we had.

I would like to express my heartiest gratitude for Miss Hana Musilova for her support, nice cooperation and for being there whenever I needed something. She never refused me a single task ever. I also wish to thank all the faculty members of the Department of Material Engineering for making me feel comfortable during my research.

I would also like to thank the worthy Vice Chancellor of BUITEMS for not only giving me this opportunity to get this degree but also providing the necessary financial support. I would also like to thank all my friends who have supported me during this time.

My deepest appreciation and gratitude is reserved for my parents and my family, for all their love and support. I want to thank my Father for giving me the strength and support throughout my studies and especially during those hard and trying times when courage dwindled. Special regards for my sweet mother who had been praying all the time for the completion of my task. I am very much thankful to my brother Zaheer for taking care of my wife, kids and family during my absence.

Finally, very special appreciations go to my beloved Saira for all her seemingly limitless love, patience, the invaluable support and her unwavering faith in me during the last 5 years. A warm and sincere ‘thanks’ to my kids, Aun and Fatima, for their love, support, and encouragement that enabled me to accomplish this milestone in my career. Thanks Fatima for your offer to write half of my thesis and thank you Aun for your patience to play with your ‘toy’.

Abbreviations

AChE	Acetylcholinesterase
AUC	Area Under the curve
ACh	Acetylcholine
ATCh	Acetylthiocholine
ANOVA	Analysis of variance
AGA	Algae Growth Analyzer
AOACI	Association of Official Analytical Chemists International
BTA	Biosensor Toxicity Analyzer
CNT	Carbon nanotube
CID	Collision-induced dissociation
CDB	Compound Database (pesticide)
Codex	Codex Alimentarius
CNS	Central nervous system
ECD	Electron capture detection
EI	Electron ionization
EPA	Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas Chromatography coupled to triple Quadrupole Tandem mass spectrometry
HPLC	High-performance liquid chromatography
IFOAM	International Federation of Organic Agriculture Movements
I %	Inhibition Percentage
IUPAC	International Union of Pure and Applied Chemistry
ISTD	Internal standard
LOD	Limit of detection
LOQ	Limit of quantitation
MT-1	Mini Thermostat
MS/MS	Tandem mass spectrometry
MRMs	Multiresidue methods
MRL	Maximum residue limit
NOP	National Organic Standards
NK	Negative Control
NIST	National Institute of Standards and Technology
OECD	Organization for economic Cooperation and Development
OP	Organophosphorus
OC	Organochlorine
PNS	Peripheral nervous system
PK	Positive Control
PCB	Polychlorinated biphenyl
(Q)	Single quadrupole
(QqQ)	Triple quadrupole
RSD	Relative standard deviation
RT	Retention Time
S/N	Signal to Noise
SPME	Solid-phase micro extraction
SRM	Selected reaction monitoring
TCh	Thiocholine
TOF-MS	Time of Flight mass spectrometer
USE	Ultra Sound Assisted Extraction
WHO	World Health Organization

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Table of Contents

List of Figures

List of Tables

Chapter 1: General Introduction

1.1 An overview of the current state of the problem

Cotton not only produces the natural fibers used in textiles and clothing but also yields a high grade vegetable oil [1]. Cotton today provides almost 38% of the world textile consumption, second only to polyester, which recently took the lead [2]. Cotton production is highly technical and difficult because of pest pressures and environment, e.g. drought, temperature and soil nutritional conditions. The total area dedicated to cotton production accounts approximately 2.4% of arable land globally and cotton accounts for an estimated 16% of the world's pesticide consumption [3].

Pesticides are widely used for the control of weeds, diseases, and pests all over the world, mainly since after Second World War, and at present, around 2.5 million tons of pesticides are used annually and the number of registered active substances is higher than 500. Humans can be exposed to pesticides by direct or indirect means. Direct or primary exposure normally occurs during the application of these compounds and indirect or secondary exposure can take place through the environment or the ingestion of food [4].

This is why development of natural biological methods of insect control was initiated. Cotton grown without the use of insect control was initiated. Cotton grown without the use of any synthetically compounded chemicals (i.e. pesticides, fertilizers, defoliants, etc.) is considered as ''organic'' cotton. It is produced under a system of production and processing that seeks to maintain soil fertility and the ecological environment of the crop [5].

Pesticides are toxic compounds that may cause adverse effects on the human and the environment. Benzoylureas, carbamates, organophosphorus compounds, organochlorine, pyrethroids, sulfonylureas and triazines are the most important groups [6]. As the pesticide residue is a potentially serious hazard to human health, the control and detection of pesticide residue plays a very important role in minimizing risk. Many methods have been developed in the last few years for the detection of pesticides. The most widely used methods are gas chromatography (GC), high-performance liquid

chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), immune assay and fluorescence. However, these techniques, which are time consuming, expensive and require highly trained personnel, are available only in sophisticated laboratories [7].

The organophosphates and carbamates are powerful inhibitors of Acetylcholinesterase [8]. They can irreversibly inhibit Acetylcholinesterase (AChE) which is essential for the function of the central nervous system [9], resulting in the buildup of the neurotransmitter acetylcholine which interferes with muscular responses and in vital organs produce serious symptoms and eventually death [10].

Biosensors based on the inhibition of Acetylcholinesterase (AChE) have been widely used for the detection of Organophosphorus (OP) compounds [11]. Electro analytical sensors and biosensors provide an exciting and achievable opportunity to perform biomedical, environmental, food and industrial analysis away from a centralized laboratory due to their advantages such as high selectivity and specificity, rapid response, low cost of fabrication, possibility of miniaturization and easy to integrate in automatic devices [12]. Electrochemical biosensors for measurement of these pesticides are based on the inhibition of AChE and the inhibition degree is proportional to the pesticide concentration [13]. Inhibition of AChE by any xenobiotic compound is used as a tool for assessment of toxicity of some pesticides such as organophosphates and carbamates [14].

Assessment of human exposure to pesticides and other toxicants through biological monitoring offers one means to evaluate the magnitude of the potential health risk of these chemicals [15]. Algae occupy an important position as the primary producers in aquatic ecosystems and they are the basis of many aquatic food chains. For this reason, they are used in environmental studies for assessing the relative toxicity of various chemicals and waste discharges [16]. Algae possess a number of distinct physical and ecological features and their ability to proliferate over a wide range of environmental conditions reflects their diversity [17, 18].

The action of toxic substances on algae is therefore not only important for the organisms themselves, but also for the other links of the food chains [19]. Algal toxicity tests and Life-cycle toxicity tests are increasingly being used in bioassay test batteries and it has been observed in several studies that for a large variety of chemical substance algal tests are relatively sensitive bioassay tools [20, 21].

The Gas Chromatography has been the predominant tool in pesticides multiresidue methodology for over 30 years. It has been widely used for the detection of pesticide residues exhibiting high stability and low polarity [22].

Several multiresidue methods for determination of organophosphorus, organochlorine and organonitrogen pesticides in crops using gas chromatography for separation of individual compounds, followed by detection with selective and sensitive detectors (ECD, NPD, FPD, AED or MS) have been proposed.

Mass spectrometry is a very sensitive and selective technique for both multiresidue determination and trace-level identification of a wide range of pesticides [23]. Confirmation of identity of pesticide residues may be performed by GC coupled with mass spectrometry (GC-MS) [24].

GC/MS/MS allows performing two consecutive stages of mass fragmentation in which parent ions fragmenting into daughter ions are monitored. This substantially improves selectivity and sensitivity of the determination compared to single-stage MS thanks to elimination of isobaric interferences and reduction of the chemical noise. Employing either of these techniques at the final determinative step is one of the most distinctive trends in pesticide residue analysis and is considered as a practical way to get around difficulties in target analytes identification in the case of difficult food and feed matrices containing excessive amounts of potentially interfering substances [25].

Unquestionably, tandem mass spectrometry (MS/MS) gives much higher degree of certainty in analyte identification than any single stage mass spectrometry technique, because isobaric interferences are avoided and multiple-component spectra can be resolved. Thanks to this, the confirmation of target analytes can be achieved with higher level of confidence. Among the different mass analyzers that can perform tandem mass spectrometry, triple quadrupole mass spectrometers have recently been proposed for the determination of pesticide residues in crops [26].

1.2 Research Objectives

The research is focused on the identification of residual hazardous compounds on cotton fibers. The thesis is divided into two main segments. One is qualitative and the other is quantitative analysis. For qualitative analysis, two different techniques have been approached.

1.2.1 Method Development utilizing Biosensors

The major intention is the development of method based on the measurement of bio-electrical signals caused by enzymatic inhibition of Acetylcholinesterase to identify residual pesticides. The objective of this research is to measure the performance of biosensor responsible for evaluation of the signals by the interaction of biological substances and residues on cotton. The performance parameters and optimization of these parameters to evaluate such a biosensor have also been determined.

1.2.2 The Impact of pesticides on the life cycle of Algae utilizing AGA

This method is dependent on the measurement of life cycle responses following exposure in microorganisms with the help of Algae Growth Analyzer (AGA). These responses can be predictive for human health evaluation on the basis of the weight of evidence which include data from all of the hazard assessment and characterization studies. Simple and quick sample preparation methods are supposed to conduct through techniques which involve extraction, enrichment and cleanup steps to obtain a homogeneous and representative final extract so as to have a worthy and reliable detection of hazardous compounds.

1.2.3 Estimation of residual hazardous compounds with GC-MS/MS

Finally, Gas Chromatography coupled to quadrupole tandem mass spectrometry is used not only for identification but also for the quantification of the analytes present in the samples. The aim is to build up a procedure with the consideration of all the crucial parameters essential for the development of an analytical method recommended by the official authorities. Both External and Internal standard approaches have been exercised. The limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy have been worked out to have a trustworthy conclusion of the anlytes present in cotton samples.

Chapter 2: Literature Review

2.1 Introduction

Along with food and shelter, clothing is one of the primary requirements of human beings. The first materials used for clothing were fur, hide, skin, and leaves. All of them were sheet like, two dimensional structures, not too abundantly available and somewhat awkward to handle. A few thousand years ago, a very important invention was made to manufacture two-dimensional systems – fabrics - from a simple mono-dimensional element - fibres. It was the birth of the textile industry based on fibre science and technology. Fibres abound in nature; they came from animals (wool, hair, silk etc.) or from plants (cotton, flex, hemp, reeds, etc.). Amongst these natural fibres, cotton is the most used fibre until today.

2.2 General Description of Cotton

Cotton has been part of the fabric of human existence for thousands of years. The uniqueness and diversity of cotton ensures this crop's enduring importance and consistency in the world markets well into the 21st century. Cotton not only produces the natural fibres used in textiles and clothing but also yields a high grade vegetable oil [1]. Cottonseed oil is recovered from cottonseed by mechanical pressing, by solvent extraction, or by a combination of the two approaches. It is used as cooking oil and in the formulations of shortening and spreads because it forms small β´–type crystals that impart a smooth consistency to solid fat products [27].

Cotton fibres are the purest form of cellulose, nature's most abundant polymer [28]. The cotton plant is a tree or a shrub that grows naturally as a perennial, but for commercial purposes it is grown as an annual crop. Botanically, cotton bolls are fruits. Cotton is a warm-weather plant, cultivated in both hemispheres, mostly in North and South America, Asia, Africa, and India.

Each cotton fibre is a single, elongated, complete cell that develops in the surface layer of cells of the cotton seed. The mature cotton fibre is actually a dead, hollow, dried cell wall. In the dried out fibre, the tubular structure is collapsed and twisted, giving cotton fibre convolutions, which differentiate cotton fibres from all other forms of seed hairs and are partially responsible for many of the unique characteristics of cotton [29].

2.2.1 Production of Cotton

Cotton is one of our favourite fibers and represents almost 38% of the world's textile consumption, second only to polyester, which recently took the lead. Cotton cultivation supports about 30 million farmers worldwide, 80% of which live in developing countries, working as smallholders [30]. The total area dedicated to cotton production accounts approximately 2.4% of arable land globally and this has not changed significantly for about 80 years [3]. Raw cotton is exported from about 57 countries and cotton textiles from about 65 countries [29]. Cotton is produced in more than 100 countries with almost 85% of all cotton produced in 7 countries as shown in Table 1, taken from the current statistics of United States Department of Agriculture (USDA).

Table 1: Cotton World Supply Use and Trade (1000 MT) (updated on 9/12/2013) [31]

Production	**2009/10**	**2010/11**	**2011/12**	**2012/13**	**Aug 2013/14**	**Sep 2013/14**
China	6967	6641	7403	7620	7185	7185
India	5182	5748	5987	5770	6096	6314
United States	2654	3942	3391	3770	2842	2808
Pakistan	2012	1881	2308	2025	2112	2112
Brazil	1187	1960	1894	1263	1524	1568
Australia	386	914	1196	1002	980	980
Uzbekistan	849	893	914	980	925	925
Other	3006	3350	4152	3923	3675	3674
Total	22243	25328	27246	26353	25340	25566

2.2.2 The Origin and Evolution of Gossypium

Cotton fibers are seed hairs from plants of the order Malvales, family Malvaceae, tribe Gossypieae, and genus Gossypium. Botanically, there are four principal domesticated species of cotton of commercial importance: hirsutum, barbadense, aboreum, and herbaceum. Each one of the commercially important species contains many different varieties developed through breeding programs to produce cottons with continually improving properties (e.g., faster maturing, increased yields, and improved insect and disease resistance) and fibers with greater length, strength, and uniformity.

Gossypium hirsutum, a tetraploid, has been developed in the United States from cotton native to Mexico and Central America and includes all of the many commercial varieties of American Upland cotton. The staple lengths of the Upland cotton fiber vary

from about 22–36 mm and the micronaire value ranges from 3.8 to 5.0. Fiber from G. hirsutum is widely used in apparel, home furnishings, and industrial products.

Gossypium barbadense, a tetraploid, is of early South American origin and provides the longest staple lengths. The fiber is long and fine with a staple length usually greater than 35 mm and a micronaire value of below 4.0. Commonly known as extra long staple (ELS). Egypt and Sudan are the primary producers of ELS cottons in the world today. Pima, which is also ELS cotton, is a complex cross of Egyptian and American Upland strains and is grown in the western United States, as well as in South America. This fiber from G. barbadense is used for the production of high quality apparel, luxury fabrics, specialty yarns for lace and knitted goods, and sewing thread.

The other commercial species--*Gossypium aboreum* and *Gossypium herbaceum*, both diploids are known collectively as ‘‘Desi’’ cottons, and are the Asiatic or Old World short staple cottons. These rough cottons are the shortest staple cottons cultivated ranging from 9.5-19 mm and are coarse (micronaire value greater than 6.0) compared with the American Upland varieties. Both are of minor commercial importance worldwide but are still grown commercially in Pakistan and India. G. aboreum is also grown commercially in Burma, Bangladesh, Thailand, and Vietnam [29].

2.2.3 Biosynthesis of Cotton

Cotton fibers are the largest (longest) single cells in nature. The fibers are single-celled outgrowths from individual epidermal cells on the outer integument of the ovules in the cotton fruit [3]. As described in [32] four overlapping but distinct stages are involved in cotton fiber development:

1. Initiation: beginning epidermis cells from ovule surface
2. Elongation: primary walls are developed
3. Secondary wall thickening and maturation
4. Desiccation: removal of moisture takes place and resultantly fiber collapses

The changes that occur in stage (4) are critical to the physical properties and use of cotton fibers; for example, twisting or formation of convolutions of the fiber upon drying increases elongation to break and aids spinning into composite yarns. However, the occurrence and periodicity of the twists are determined by the fiber structure that was formed by active cellular processes in the first three stages.

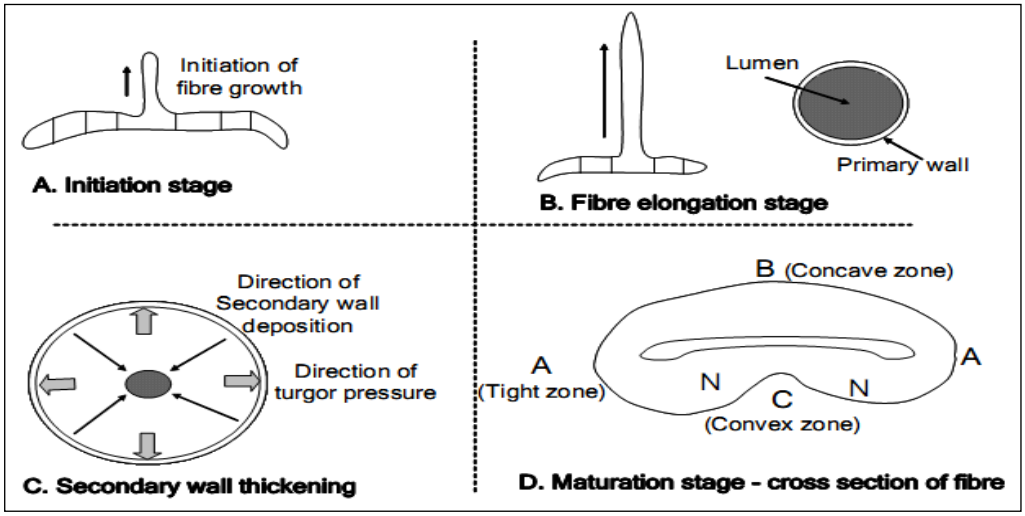


Figure 1: An illustration of various stages of cotton fibre growth [33]

Initiation: refers to the ballooning out of the fiber initial above the seed epidermal surface on the day of flowering (Fig 1A, Fig 2a), or anthesis. Customarily fiber age is described by days post-anthesis, DPA. Each individual fiber remains in the initiation stage with a bulbous tip for about two days.

Elongation: can be defined as beginning when the individual fiber develops a sharply tapered tip (Fig 2b and 2c). This stage is characterized by rapid primary cell wall synthesis as the single-celled fiber attains lengths that can be greater than 2.25 inches. Elongation continues until 14 to 40 DPA with the duration dependent upon genotype and environment [34]. Cell elongation is crucial for fibre growth and development and determines the length and fineness of the fibre. Cotton fibres are unicellular so there is no cell division [35]. Figure 1B schematically shows the growth of a cotton fibre.

Thickening: begins when the cell wall starts to thicken. The times of initiation and duration of this phase also depend on genotype and environment. Generally, thickening begins between 12 and 20 DPA while elongation continues [34]. Cell elongation and secondary wall thickening are overlapping stages in the cotton fibre development [35]. Cell wall thickening begins with deposition of a thicker primary wall, but soon the deposition of a cellulose-rich secondary wall begins. The cellulose-rich secondary wall forms the bulk of the mature fiber, and its deposition is completed by 35 to 55 DPA. The secondary wall is deposited from the outside to the centre of the fibre as shown in Figure 1C. The secondary wall of the cotton fiber is the purest cellulose structure produced in bulk by higher plants, containing more than 95% cellulose [34].

(a) (b) (c)

Figure 2: Scanning electron micrographs of seed with the initiation (a) 0 dpa of fibers and the beginning of elongation (b), 1 dpa ; (c) 2 dpa [28]

Maturation: After completion of secondary wall thickening, the capsule breaks, opens and the young fibres undergo a drying process. Until this stage, the cotton fibre has a cylindrical shape. Removal of water from the fibre causes the internal layer to twist and collapse producing wrinkles and moulds to the under laying layers. Figure 1D shows the schematic representation of collapsed cotton fibre. The cross section of a mature dry fibre has a convex and concave side [36]. The fully hydrated cylindrical fibers are cylindrical under light microscopy (Fig 3a). The fluid loss from the lumens causes the cylindrical fibers to collapse to form twists or convolutions (Fig 3b). The matured fibers dry into flat twisted ribbon forms (Fig 3c). The twist or convolution directions reverse frequently along the fibers. The convolution angle has been shown to be variety dependent [28].

Figure 3: Light micrographs of fully hydrated fibers (left), dried fibers (middle) and mature fibers (right) [28]

Mature fibers can be easily detached from the seeds. After detachment of longer fibers (lint fibers), the seeds of many cultivars remain covered by many short fibers, the so called fuzz. Some cultivars have naked seeds, that is; fuzz fibers are missing [37].

2.2.4 Cotton fibre structure

The cell wall is a dynamic structure which composition and form can change markedly, not only during cell growth but also after the cells have become matured [38]. The cotton fibre is structurally built up into concentric zones and a hollow central core known as the lumen. The mature fibre essentially consists of (from outside to inside) the cuticle i.e. the outermost layer, the primary cell wall, the secondary wall and the lumen [38, 39].

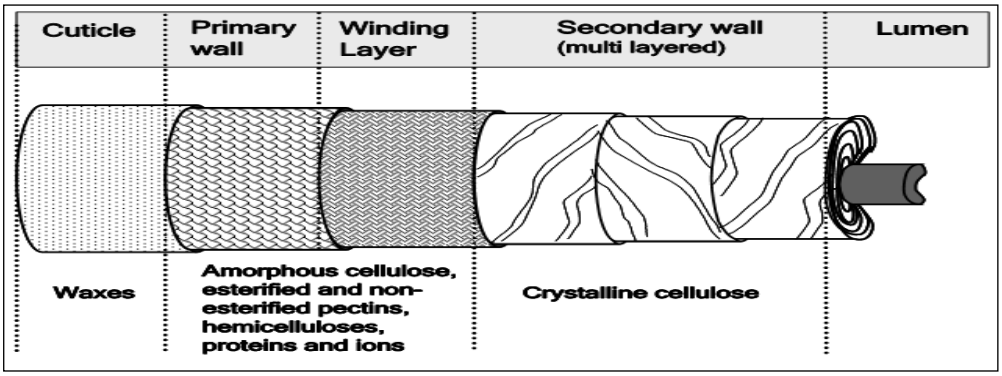


Figure 4: A schematic representation of mature cotton fibre showing its various layers [33]

Figure 4 systematically shows the different layers present in the cotton fibre with the compositions of each layer. Cotton contains nearly 90% of cellulose and around 10% of non-cellulosic substances, which are mainly located in the cuticle and primary wall of the fibre. Typical components in dry mature cotton fibres are given in Table 2. From this table it is clear that most of the non-cellulosic materials are present in the outer layers of cotton fibre.

Table 2: Typical composition of dry mature cotton fibre [38]

Constituents	**Composition (%)**	
	Whole fibre	**Outer layer**
Cellulose	94	54
Protein (Nitrogen Substances)	0.6-1.3	8
Pectic substances	0.9-1.2	9
Ash	1.2	3
Waxes	0.6-1.30	14
Organic acids	0.8	-
Others	1.4	12

Figure 5 illustrates schematically the distribution of cellulose and other noncellulosic materials in the various layers of cotton fibre.

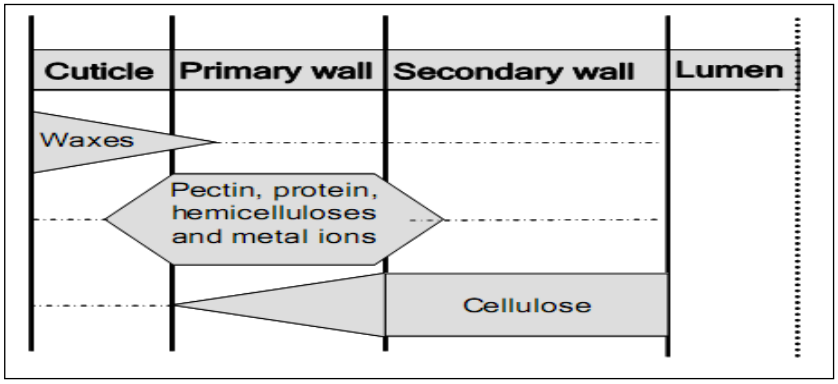


Figure 5: A schematic representation of the cellulosic and non-cellulosic materials in the fibre [33]

Regarding the degree of fibre maturity, cotton fibres are simply classified into two categories of immature and mature fibres. A typical cross-section overview of an immature (left) and a mature (right) single fibre is shown in Figure 6. Obviously, the ratio of the secondary wall to the total area of the primary wall and lumen increases with the secondary wall thickening (or fibre maturity) [28, 40, 41].

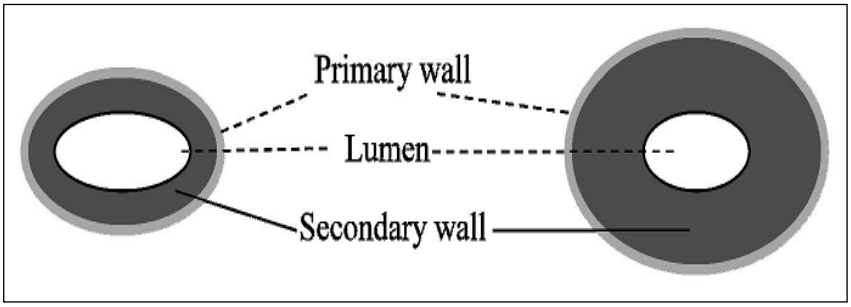


Figure 6: Overview of an immature (left) and a mature (right) single fibre [40]

2.2.5 Pests and diseases

Cotton is highly susceptible to pests, especially in humid areas [3]. Worldwide 15% of cotton yield loss is due to insect damage [42].

Pest infestation is a major destabilizer of cotton production. The significance of pest control can be gauged by the fact that cotton accounts for 22.5% of all root insecticide sales worldwide. Cotton insects are classified into following two groups on the basis of feeding behavior.

Sucking pests

This group includes jassids (Amrasca bigutulla bigutulla), whitefly (Bemisia tabaci), aphids (Aphis gossypii), thrips (Thrips tabaci) and mites (Tetranynchus sp).

Tissue feeders

This group includes bollworms and weevils including American bollworms (Helicoverpa armigera and H. virescens), pink bollworm (Pectinophora gossypiella), spotted bollworms (Earias vitella and E. inbsulana), tobacco cut worm (Spodoptera litura), bollweevil (Anthonomous grandis), red bollworm (Diparopsis castanea) and shoot weevil (Alcidodes affaber).

In general losses due to sucking pests (5%– 10%) are much less than from bollworms (25%–50%). During reproductive period, bollworms not only cause reduction in the yield but also affect fiber properties. Sucking pests, active during reproductive period, are vectors for many pathogen and viruses; the best example is white fly, the vector for cotton leaf curl virus [43]. In general fungal, viral and bacterial plant pathogens as well as nematodes are of lesser importance in cotton cultivation than insects [42].

2.3 Pesticides

Cotton is considered to be quite a difficult crop to grow because it is sensitive to drought, low temperatures and attacks by various insects. The cultivation of cotton has been estimated to consume 11% of the world's pesticides while it is grown on just 2.4% of the world's arable land [44].

Pesticides are chemicals used to manage pest organisms in both agricultural and non agricultural situations. By definition, a pesticide is a *"substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm or otherwise interfering with the production, processing, storage, transport, or marketing of food, agricultural commodities, wood, wood products or animal feedstuffs, or which may be administered to animals for the control of insects, mites/spider mites or other pests in or on their bodies"* [45].

The term pesticide covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others. The pattern of pesticide usage in the world can be seen in Figure 7 [46].

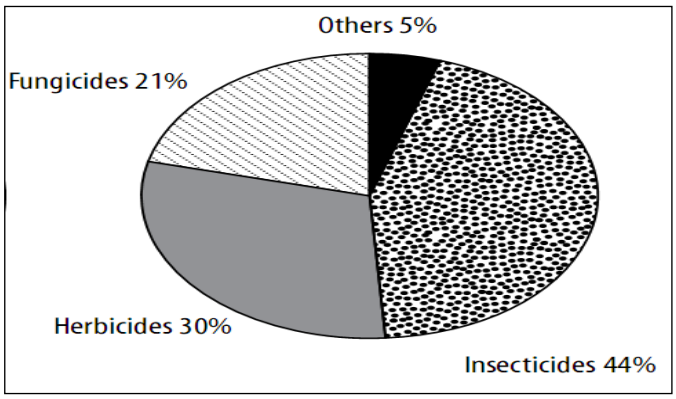


Figure 7: Consumption pattern of pesticides [46]

2.4 Classification of Pesticides

Pesticides are classified in a number of different ways (e.g., by chemistry, mode of action or type of application). Although pesticides of similar chemistry often share similar target sites, this is not always the case, as some pesticides with common structural features have varying toxicities to widely divergent organisms. For example, several insecticidal dithiocarbamates are not herbicidal and vice versa.

However, structurally similar pesticides often impart similar toxicological actions on non target organisms. Table 3 shows a brief picture of categories of the pesticides.

Table 3: A selection of commonly used pesticides [47]

Fungicides	Herbicides	Insecticides
Inorganics Sulfur Copper Sulphate	*Triazines* Atrazine Cyanazine	*Chlorinated hydrocarbons* DDT Endosulfan
Substituted aromatics Chlorothalonil PCNB	*Chloroacetamides* Alachlor Metachlor	*Organophosphates* Parathion Chlorpyrifos Terbufos Phorate Malathion Acephate
Dithiocarbamates Mancozeb Maneb Ziram	*Chlorophenoxy* 2,4-D 2,4,5-T	
Dicarboximides Captan	*Nitroanilines* Trifluralin Pendimethalin	*Carbamates* Carbofuran Carbaryl Aldicarb
Benzimidazoles Benomyl	*Phosphono amino acids* Glyphosate	
	Thiocarbamates EPTC	*Pyrethroids* Permethrin Cypermethrin Cyhalothrin

Fungicides include a variety of chemicals ranging from the typically broad-spectrum metallic fungicides to pest-specific organic compounds. Classes of fungicides include copper and sulphur based fungicides, dithiocarbamates, aromatic hydrocarbons, benzimidazoles, phenylamides, and triazoles.

Herbicides are typically classified by chemistry as inorganic salts, halogenated alkanoic derivatives, (aryloxy) alkanoic acids, arylcarboxylic acids, esters of 2- [4-(aryloxy) phenoxy] alkanoic acids, nitriles, amides, anilides, phenols, diphenyl ethers, 2,6-dinitroanilines, carbamates, thiocarbamates, ureas, sulphonylureas, imidazolinones, pyrimidines, pyridazines, 1,3,5-triazines, bipyridinium compounds, miscellaneous heterocyclic compounds, oximes, organophosphates, organoarsenicals, and soil fumigants [47].

The application of natural insecticides, primarily of plant origin, for plant protection and hygiene, preceded by a long time that of synthetic insecticides. In certain parts of Europe, plants were sprayed with an extract of tobacco plants as early as 1690. In the period between 1900 and the 1940s only nicotine, pyrethrins, rotenone and quassia were used in addition to inorganic insecticides, and their application virtually ceased on the discovery and large-scale economic production of synthetic insecticides [48].

Insecticides have been traditionally classified into four major groups; chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids. Insect growth regulators, such as methoprene, and bacterial endotoxins, such as that produced by *Bacillus thuringiensis*, were added to this list by the 1980s. Recently, several insecticides with novel modes of action have been identified (e.g., nicotinoids, fipronil, and the spinosids).

Table 4: Periods of major insecticide introductions and typical use rates, adopted from [50]

Insecticide Class	Major Introductions	Typical Use rate (Kg/ha)
Arsenical	1890-1940	4.00-60.00
Chlorinated hydrocarbon	1939-1956	1.00-4.00
Organophosphorous	1946-1986	0.50-2.00
Carbamate	1957-1984	0.50-2.00
Pyrethroid	1973-1992	0.01-0.20
Benzoylurea	1972-2002	0.01-0.05
Neonicotinoid	1990-2001+	0.01-0.10
Phenylpyrazole	1992-2001+	0.10-0.15

The number of existing active ingredients currently employed as pesticides is quite large (approx. 1000), and the great majority have been subject to extensive

toxicological and environmental testing as part of government registration processes. There has been a general trend during the past 30–40 years toward introduction of products with lower application rates, decreased environmental persistence, and reduced non target organism toxicity.

As an example of this trend, Table 4 lists major classes of insecticide products along with their period of significant introductions and typical field use rates [49].

Pesticides are intended to disrupt a primary target in the pest. The pesticides interact with a specific enzyme, receptor, protein, or membrane, initiating a series of events that is deleterious or lethal to the pest. There are a few similar targets for the various pesticide types but they are usually very different.

Most insecticides quickly disrupt neurotransmission to alter insect behaviour or survival. Rapid action is usually required because insects cause economically important damage within a few hours or days. Insecticides can be practical with only a limited biological range like aphids or caterpillars. Herbicides generally inhibit plant-specific pathways, blocking amino acid or fatty acid biosynthesis or photosynthesis to "starve" the weed over several days. Fungicides act on many basic cellular functions important to hyphal tip growth (Fig 8).

Fungi are evolutionarily far more diverse than insects or weeds. They include not only the true fungi but also the Oomycetes having motile stages and controlled by oomyceticides. There are a broad variety of fungicide targets which vary in their importance for survival [51].

Table 5: Molecular Targets of the Major Classes of Insecticides [52]

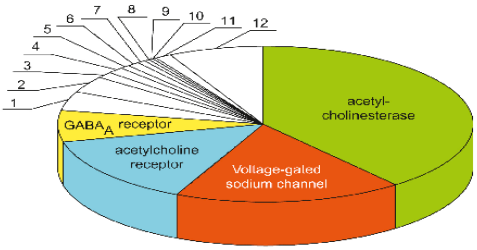
TARGET	INSECTICIDE	EFFECT
Acetylcholinesterase	Organophosphates	Inhibition
	Carbamates	Inhibition
Sodium channels	Pyrethroids (Type I and II)	Activation
	DDT	Activation
	Dihydropyrazoles	Inhibition
Nicotinic acetylcholine receptors	Nicotine	Activation
	Neonicotinoids	Activation
GABA receptors-gated chloride channels	Cyclodienes	Inhibition
	Phenylpyrazoles	Inhibition
	Pyrethroids (Type II)	Inhibition
Glutamate-gated chloride channels[1]	Avermectins	Activation
Octopamine receptors[2]	Formamidines	Activation
Mitochondrial complex I	Rotenoids	Inhibition

1 Found only in insects. In mammals avermectins activate GABAA receptors.
2 In mammals, formamidines activate alpha2-adrenoceptors.

Insecticide (mostly neurotransmission)

Other Targets

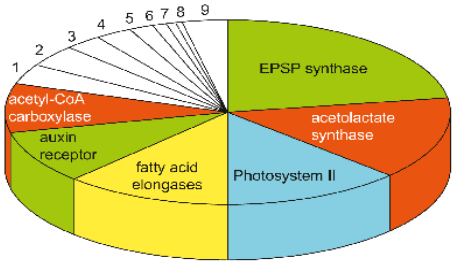
1. Chitin biosynthesis
2. Glutamate (chloride) receptor
3. Acetyl-CoA carboxylase
4. ATP synthase
5. Ecdysone receptor
6. Uncoupler
7. Bt toxin
8. NADH dehydrogenase
9. Succinic dehydrogenase
10. Octopamine receptor
11. Unspecific
12. Unknown



Herbicide (mostly plant specific pathways)

Other Targets

1. Tubulin
2. Photosystem I
3. Protoporphyrinogen IX oxidase
4. 4-hydroxyphenyl pyruvate dehydrogenase
5. Phytoene desaturase
6. Glutamine synthase
7. Others
8. Unknown
9. Unspecific



Fungicide (mostly basic cellular functions)

Other Targets

1. Succinic dehydrogenase
2. Protein His Kinase (osmo sensor)
3. RNA polymerase
4. Scytalone dehydratase
5. Sterol Δ14 reductase
6. Uncoupler
7. Methionine biosynthesis
8. Protein Kinase (osmo sensing)
9. Phospholipid biosynthesis
10. Protein biosynthesis (ribosomes)
11. Sterol 3-keto reductase
12. ATP synthase
13. Chitin biosynthesis
14. Dihydroorotate dehydrogenase
15. Inositole biosynthesis
16. Others, unknown

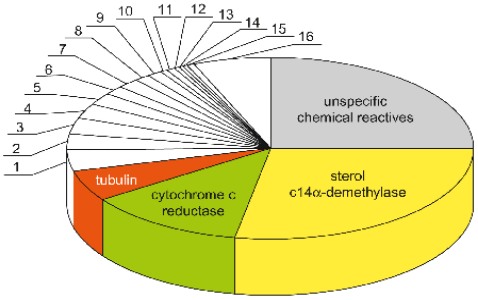


Figure 8: Insecticide, herbicide, and fungicide targets [51]

Insecticides play a most relevant role in the control of insect pests, particularly in developing countries. All of the chemical insecticides in use today are neurotoxicants, and act by poisoning the nervous systems of the target organisms (Table 5).

Insecticides have higher acute toxicity toward non target species compared to other pesticides. Some of them, most notably the organophosphates, are involved in a great number of human poisonings and deaths each year [52].

2.4.1 Chlorinated Hydrocarbon Insecticides

Although the organochlorine insecticides were widely used in agriculture and malarial control programs from the 1940s to 1960s with dramatic benefits due to their properties of low volatility, chemical stability, lipid solubility, slow rate of biotransformation, and degradation, they fell into disfavour because of their persistence in the environment, wildlife, and humans. All organochlorine insecticides can be absorbed through the skin as well as by the respiratory and oral routes, but the importance of dermal absorption varies greatly for the different compounds. The organochlorine insecticides include the chlorinated ethane derivatives, such as DDT and its analogues; the cyclodienes, such as chlordane, aldrin, dieldrin, heptachlor, endrin, and toxaphene; the hexachlorocyclohexanes, such as lindane; and the caged structures mirex and chlordecone (Fig 9).

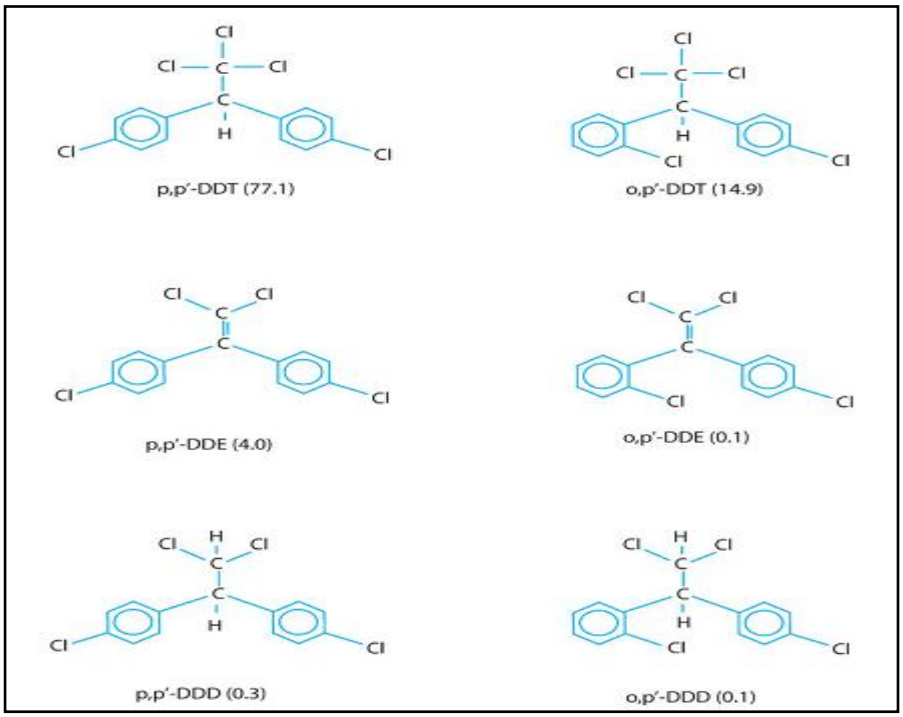


Figure 9: Structures of the organochlorine insecticide p,p´- DDT and its isomers [52]

Their acute toxicity is moderate (less than that of organophosphates), but chronic exposure may be associated with adverse health effects particularly in the liver and the reproductive system. Primarily because of ecological considerations, these compounds have been banned in most countries in the past thirty years. Yet, because of their environmental persistence and high lipophilicity, exposure to these compounds continues, most notably through the diet [52, 53]. In varying degrees, organochlorines are absorbed orally, by inhalation, and by dermal absorption [59].

Several pesticides may fall into the category of endocrine disruption, and among these, a large number are organochlorine insecticides. The o,p´-isomer of DDT, which comprise approximately 15% of the technical grade product (Fig 9), has estrogenic properties, in that it can act as an agonist at estrogen receptors (ER) α and β [52].

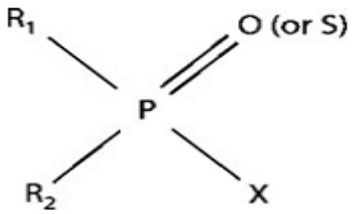
2.4.2 Organophosphate Insecticides

Although a number of organic phosphorus (OP) compounds were synthesized in the 1800s, their development as insecticides only occurred in the late 1930s and early 1940s [54, 55]. The German chemist Gerhard Schrader is credited for the discovery of the general chemical structure of anticholinesterase OP compounds, and for the synthesis of the first commercialized OP insecticide [Bladan, containing TEPP (tetraethyl pyrophosphate) as the active ingredient], and for one of the most known, parathion, in 1944. Since then, hundreds of OP compounds have been made and commercialized worldwide in a variety of formulations. More than half of the insecticides used are OPs, and some OPs are among the most extensively used pesticides. The chemistry of OPs has been thoroughly investigated [56].

The principal mechanism for the mammalian toxicity of OP insecticides is inhibition of the intended target, Acetylcholinesterase (AChE), in the peripheral and central nervous systems (PNS and CNS), respectively [57, 58]. This enzyme is critical to normal control of nerve impulse transmission from nerve fibers to muscle and gland cells, and also to other nerve cells in autonomic ganglia and in the brain. At sufficient dosage, loss of enzyme function allows accumulation of acetylcholine (ACh, the impulse-transmitting substance) at cholinergic neuroeffector junctions (muscarinic effects), at skeletal nerve-muscle junctions and autonomic ganglia (nicotinic effects), and in the brain. In the brain, high ACh concentrations cause sensory and behavioural disturbances, incoordination and depressed motor function.

Depression of respiration and pulmonary edema are the usual causes of death from organophosphate poisoning. Recovery depends ultimately on generation of new enzymes in all critical tissues. Organophosphates are efficiently absorbed by inhalation, ingestion, and skin penetration [59].

The general structure of OP insecticides can be represented by



Where X is the so-called "leaving group," that is displaced when the OP phosphorylates Acetylcholinesterase (AChE), and is the most sensitive to hydrolysis; R_1 and R_2 are most commonly alkoxy groups (i.e., OCH_3 or OC_2H_5), though other chemical substitutes are also possible; either an oxygen or a sulphur (in this case the compound should be defined as a phosphorothioate) are also attached to the phosphorus with a double bond.

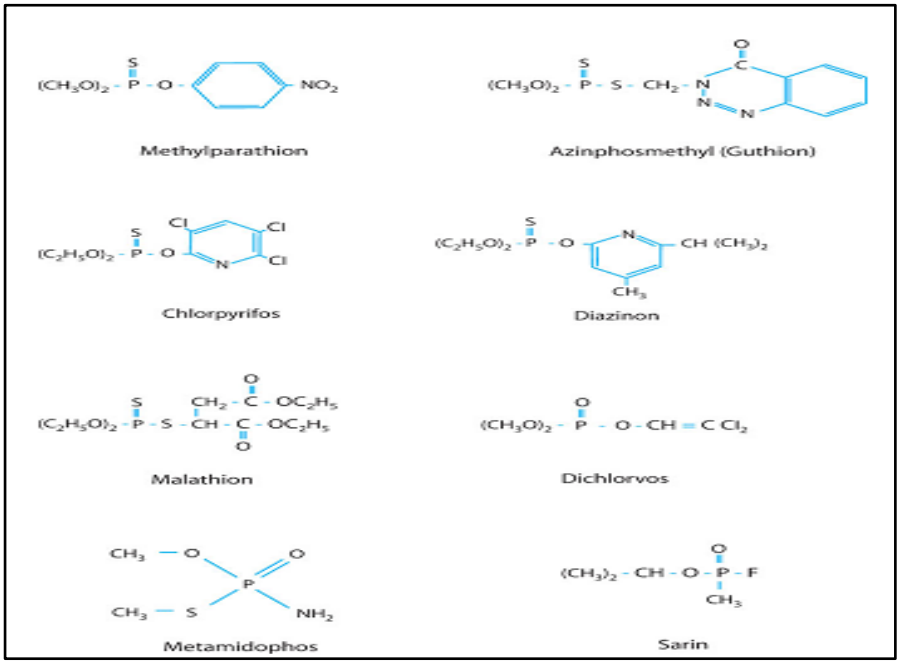


Figure 10: Structures of some organophosphorus insecticides and of the nerve agent sarin [52]

Based on chemical differences, OPs can be divided into several subclasses, which include phosphates, phosphorothioates, phosphoramidates, phosphonates, and others. Figure 10 shows the chemical structures of some commonly used OPs. Most are phosphorothioates, and need to be bio activated in vivo to their oxygen analogs to exert their toxic action, but some (e.g., dichlorvos or the nerve agent sarin) have P=O bonds. Most OPs used as insecticides have two methoxy or ethoxy side chains [48, 52, 56].

OP insecticides, e.g. acephate, chlorpyrifos, diazinon, malathion, and parathion are the most widely used agrochemicals for the control of insect pests [53, 55]

2.4.3 Carbamate Insecticides

Carbamates generally act quickly. They are strongly toxic to a wide range of insect pests, but have a weak effect on the red spider mite. Some of them exhibit systemic characteristics. The duration of their action varies considerably [48].

N-methyl carbamates are absorbed by inhalation and ingestion and some by skin penetration. Dermal absorption of particular compounds (notably carbofuran) is very slight. N-methyl carbamates are hydrolyzed enzymatically by the liver and the degradation products are excreted by the kidneys and the liver [59].

Carbamate insecticides have a variety of chemical structures (Fig 11), but all derive from carbamic acid, the majority being *N* -methylcarbamates. They present different degrees of acute oral toxicity, ranging from moderate to low toxicity such as carbaryl, to extremely high toxicity, such as aldicarb. Carbamates are susceptible to a variety of enzyme-catalyzed biotransformation reactions, and the principal pathways involve oxidation and hydrolysis [60].

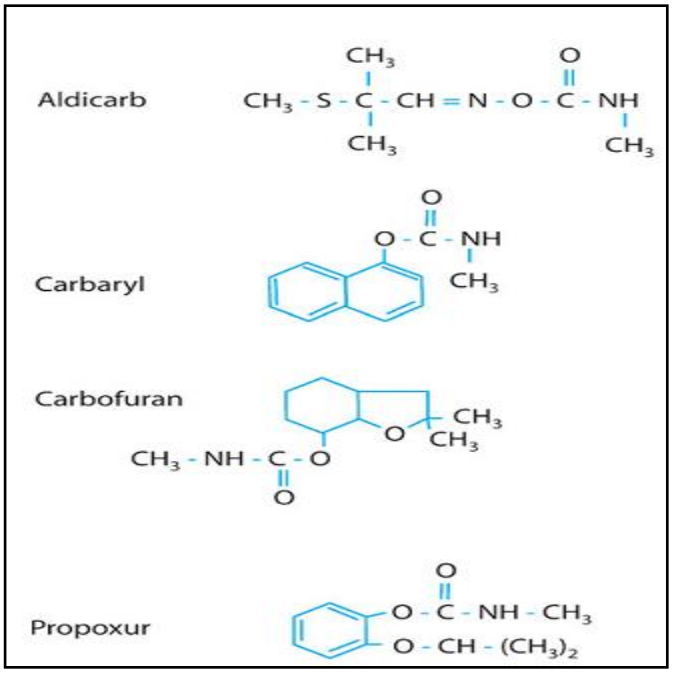


Figure 11: Structures of some carbamate insecticides [52]

The mechanism of toxicity of carbamates is analogous to that of OPs, in that they inhibit AChE. However, inhibition is transient and rapidly reversible, because there is rapid reactivation of the carbamylated enzyme in the presence of water (Table 6).

Table 6: Rates of Cholinesterase Inhibition by Carbamate and Organophosphorus Esters [61]

$$EH + AB \underset{k\text{-}1}{\overset{k1}{\rightleftarrows}} EHAB \xrightarrow{k2} EA + BH \xrightarrow{k3} EH + AOH$$

		REACTION RATES	
PARAMETER	KINETIC CONSTANT	CARBAMATE	ORGANOPHOSPHORUS
Complex formation	k_{-1}/k_1	Rapid	Rapid
Inhibition rate	k_2	Variable	Rapid to moderately rapid
Reactivation rate	k_3	Relatively rapid	Slow to extremely slow

Additionally, carbamylated AChE does not undergo the aging reaction. The sign and symptoms of carbamate poisoning are the same as observed following intoxication with OPs, and include miosis, urination, diarrhea, salivation, muscle fasciculation, and CNS effects. However, differently from OPs, acute intoxication by carbamates is generally resolved within a few hours [52].

2.4.4 Pyrethroids

Pyrethrin, the dried flower of Chrisanthenum cinerariaefolium, or its solvent extract, has been used for centuries in order to kill insects [48]. However, because pyrethrins were decomposed rapidly by light, synthetic analogs, the pyrethroids were developed. Because of their high insecticidal potency, relatively low mammalian toxicity, lack of environmental persistence, and low tendency to induce insect resistance, pyrethroids have encountered much success in the past thirty years, and now account for more than 25% of the global insecticide market.

Pyrethroids are used widely as insecticides both in the house and in agriculture, in medicine for the topical treatment of scabies and head lice, and in tropical countries in soaked bed nets to prevent mosquito bites. Pyrethroids are known to alter the normal function of insect nerves by modifying the kinetics of voltage-sensitive sodium channels, which mediate the transient increase in the sodium permeability of the nerve membrane that under lies the nerve action potential [62].

Pyrethrins are absorbed orally and by inhalation, but only slightly across intact skin. They are very effectively hydrolyzed to inert products by mammalian liver enzymes [59]. All pyrethroid insecticides contain an acid moiety, a central ester bond, and an alcohol moiety (Fig 12). The acid moiety contains two chiral carbons, thus pyrethroid typically exist as stereo isomeric compounds (*trans* and *cis*). Additionally, some pyrethroids also have a chiral carbon on the alcohol moiety, allowing for a total of eight

different stereoenantiomers. These chemical considerations are relevant, as pyrethroids' effects on sodium channels, their insecticidal activity, and their mammalian toxicity, are stereospecific. The *cis* isomers are generally more toxic than the corresponding *trans* isomers [63].

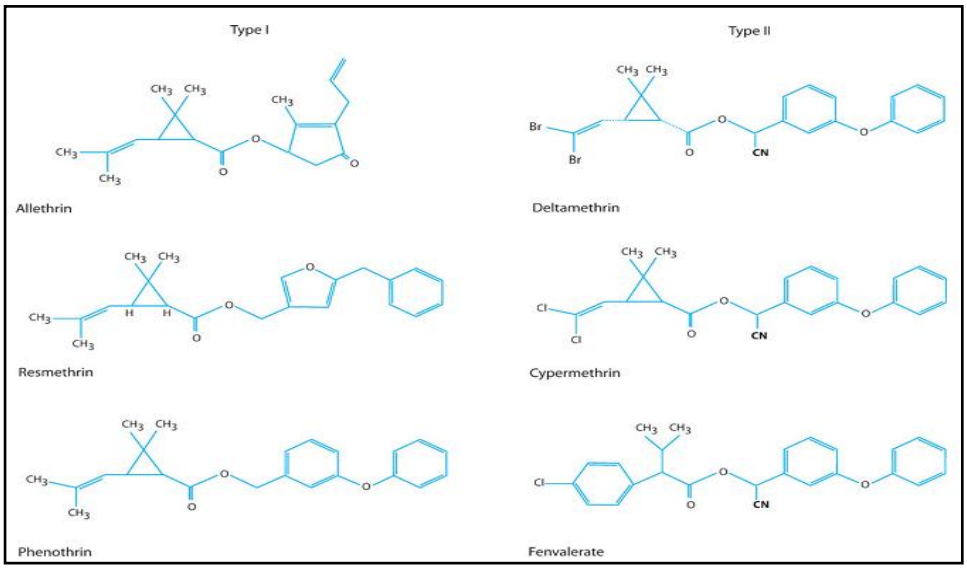


Figure 12: Structures of Type I (left) and Type II (right) pyrethroid insecticides [52]

Based on toxic signs in rats, pyrethroids have been divided into two types (Table 7). Type I compounds produce a syndrome consisting in marked behavioral arousal, aggressive sparring, increased startle response, and fine body tremor progressing to whole-body tremor and prostration. Type II compounds produce profuse salivation, coarse tremor progressing to choreoatetosis and clonic seizures [64].

Table 7: Classification of Pyrethroid Insecticides Based on Toxic Signs in Rats [64]

SYNDROME	SIGNS AND SYMPTOMS	EXAMPLES
Type I (T syndrome)	Aggressive sparring Increased sensitivity to external stimuli Whole body tremors Prostration	Allethrin Bioallethrin Resmethrin Phenothrin
Type 2 (CS syndrome)	Pawing and burrowing Profuse salivation Coarse Tremor Choreoatetosis Clonic seizures	Deltamethrin Fenvalerate Cypermethrin Cyhalothrin

A key structural difference between type I and type II pyrethroids is the presence only in the latter of a cyano group at the α carbon of the alcohol moiety of the compound (Fig 12). However, certainpyrethroids (e.g., cyphenothrin, flucythrinate) elude such classification, as they produce a combination of the two syndromes [62, 65].

2.5 Benefits of pesticides

It is well accepted that the use of any biologically active compound can be accompanied by various degrees of toxic reactions or adverse effects. Therefore, even the safe and biologically degradable pesticides may harm the end-consumers: human and animals. Although the hazards of pesticides are evident when used irrationally and excessively, their positive and essential contribution to health and economy is worth mentioning [46].

Plants are the main source of food and are liable to being attacked by a wide range of pests. Pesticides eliminate or, at least, minimize the occurrence of certain arthropods and other vector borne diseases. This, in turn, increases the production of plant-based food and fibers [22]. This higher yield might bring additional revenue that could be put towards children's education or medical care, leading to a healthier, better educated population.

Tremendous benefits have been derived from the use of pesticides in forestry, public health and the domestic sphere and, of course, in agriculture. Food grain production in India, which stood at a mere 50 million tons in 1948–49, had increased almost fourfold to 198 million tons by the end of 1996–97. Similarly outputs and productivity have increased dramatically in most countries, for example wheat yields in the United Kingdom, corn yields in the USA. Increases in productivity have been due to several factors including use of fertiliser, better varieties and use of machinery. Pesticides have been an integral part of the process by reducing losses from the weeds, diseases and insect pests that can markedly reduce the amount of harvestable produce. It is stated [66] that "considerable economic losses" would be suffered without pesticide use and quantified the significant increases in yield and economic margin that result from pesticide use. Moreover, in the environment most pesticides undergo photochemical transformation to produce metabolites which are relatively non-toxic to both human beings and the environment [67].

2.6 Human Exposure and Risk

Of the many possible negative effects of pesticide use, the impact on human health remains a major concern [68]. Human exposure to pesticides can be described in several ways, e.g., acute or chronic, occupational or non occupational, intentional or unintentional, accidental or incidental. Within each type the exposure can be oral (by

mouth), respiratory (by inhalation), or dermal (through the skin). The different categories of exposure were depicted in Figure 13. Since individuals are often exposed in more than one way, the total exposure from all sources needs to be considered in assessing the health risk [69].

Although the general public appear to regard exposure to pesticides from residues in food and, perhaps, water of greatest concern, there are multiple other sources of exposure which can compound with those from residues. These include hand to mouth contact from pesticides used within buildings, veterinary medicines used against domestic pets (e.g. flea sprays), and contamination of food and working surfaces from the residential use of pesticides (e.g. control of insects). Thus, although the oral route is probably the major route of exposure for the general public, the skin and eyes probably are also significant and inhalation the least [53].

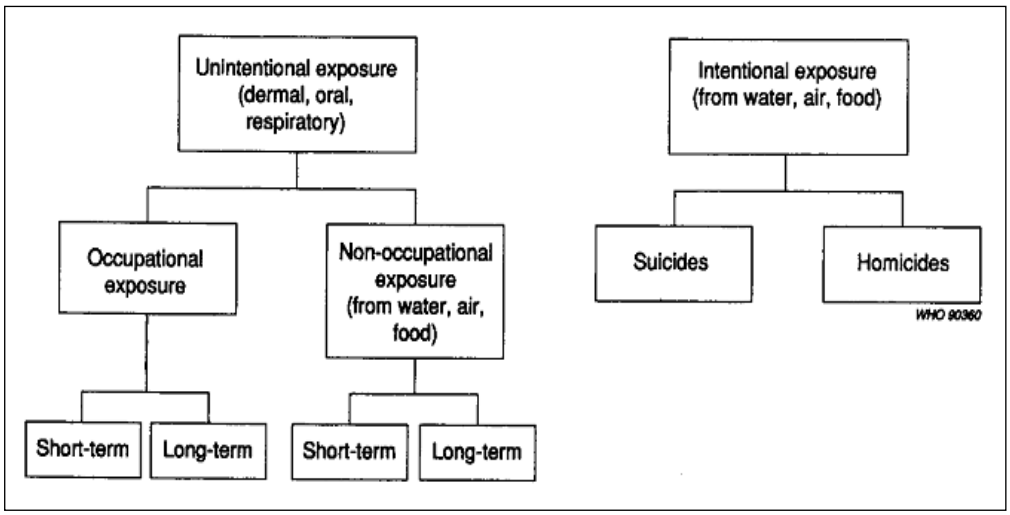


Figure 13: Types of exposure to pesticides [69]

Among the 74 active ingredients listed in Class1A (Extremely hazardous) and Class 1B (Highly hazardous), 48 (65%) are insecticides, in particular organophosphates (Table 8). Rodenticides are also highly toxic to rats, but do not present the same hazard to humans. Indeed, warfarin, one of the most widely used rodenticides, is the same chemical used as an effective "blood thinner" (anticoagulant) for prevention of stroke and other blood clot related conditions. Herbicides, again as a class, have generally moderate to low acute toxicity, one exception being paraquat (which has a low dermal toxicity but causes fatal effects when ingested). Fungicides vary in their acute toxicity, but this is usually low [52].

Table 8: WHO-Recommended Classification of Pesticides by Hazard [70]

WHO Class		LD_{50} for the rat (mg/kg body weight)	
		Oral	Dermal
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5–50	50–200
II	Moderately hazardous	50–2000	200–2000
III	Slightly hazardous	Over 2000	Over 2000
U	Unlikely to present acute hazard	5000 or higher	

Pesticides are not always selective for their intended target species, and adverse health effects can occur in non target species, including humans. In the general population and in occupationally exposed workers, a primary concern relates to a possible association between pesticide exposure and increased risk of cancer [71, 72], and Parkinson's disease [73]. Evidence that some pesticides may act as endocrine disruptors, possibly contributing to various adverse effects in humans, including cancer and reproductive and developmental toxicity, has also prompted additional concerns and initiatives [74]. Populations living near agricultural fields are at higher risk of exposure due to their proximity to areas where pesticides are frequently applied [75, 76]. Yet, from a global perspective, the major problem with pesticides remains that of acute human poisoning [52].

The WHO (1990) estimates an annual incidence of unintentional acute poisoning of about one million, with an overall mortality rate of about 1% (of which only 1% is in developed countries) [69]. The majority of unintentional pesticide poisonings are occupational. Population-based studies in 17 countries gave annual incidence rates of unintentional pesticide poisoning of 0.3–18 per 100,000 [77]. The estimated annual incidence of intentional single exposure poisoning is about two million with a 5.7% mortality rate [69]. Such poisoning is more frequent in developing than in developed countries. In Indonesia, Malaysia and Thailand suicide attempts (usually with organophosphorus compounds) represent 60–70% of acute pesticide poisonings [77, 79]. Acute pesticide poisoning rates in a highland potato-growing area of Ecuador were 171 per 100,000 inhabitants, in the period 1991-92, most of which were occupational [79]. Observed mortality rates of 21 per 100,000 are among the highest reported anywhere in the world. Thailand monitors blood cholinesterase levels in several hundred thousand farmers every year. In the period 1992 – 2000, 13 to 25% of farmers

had cholinesterase levels that were considered abnormal, indicating excessive exposure to organophosphate or carbamate pesticides [69, 80].

WHO estimated approximately 20,000 workers die from exposure every year, the majority in developing countries [81]. The number of intoxications with organophosphates is estimated at some 3000,000 per year and the number of deaths and casualties some 300,000 per year [82]. Ahmed and co workers have reported 64 percent of fatal cases of acute pesticides poisoning in Multan, Pakistan occurred due to OPs pesticide spraying [83, 84].

The most obvious danger to human health from pesticides is through accidental poisonings. Chronic illness appears to arouse the greatest concern, especially the possibility of harm to children. What seems to worry people more is that long-term exposures to extremely small quantities of pesticides may be dangerous [85].

2.7 Impact on environment

Multiple reports exist on the unwanted side-effects of pesticides on wildlife. Over-spraying, accidents and aerial spraying are the most significant events affecting the environment. Pesticides applied in cotton production have also been documented as adversely affecting river ecosystems in Australia, leading to lower quantities and lessened diversity of water organisms [42].

In many respects, the greatest potential for unintended adverse effects of pesticides is through contamination of the hydrologic system, which supports aquatic life and related food chains and is used for recreation, drinking water, and many other purposes. Water is one of the primary mechanisms by which pesticides are transported from targeted application areas to other parts of the environment (Fig 14) and, thus, there is potential for movement into and through all components of the hydrologic cycle [86]. Pesticides move through air, soil, and water, finding their way into living tissues where they undergo biological magnification. Thus, the deterioration of the ecosystem by the continuous use of fertilizers and pesticides has been observed. The quality of river water deteriorates almost as soon as it enters the plains. Chemical fertilizers and pesticides constitute one of the major pollutants of the river. Although the water is treated before supply, the treatment does nothing to remove the pesticide traces and industrial pollutants present in the water. The burden of waterborne disease is about 30.5 million of DALYs (disabled life years).

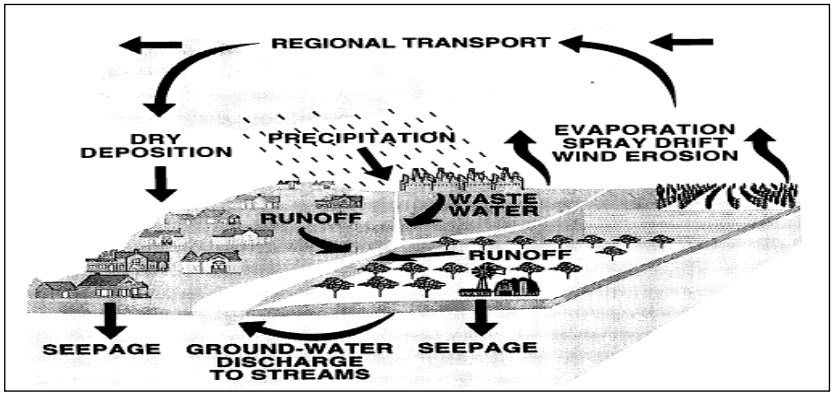


Figure 14: Pesticide movement in the hydrologic cycle [86]

Leaching from agricultural fields has been the most important nonpoint source of pollution to the aquatic environment (Fig 15). Traces of HCH and DDT have been found in rivers of the United States and Europe, where they have been banned for more than two decades [22].

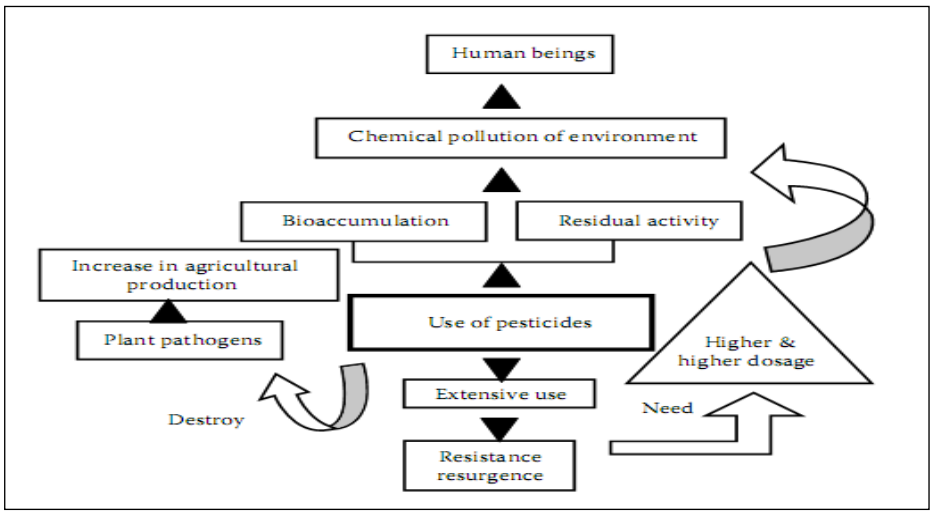


Figure 15: Today's realization [22]

In the EU, numerous ground water supplies now exceed the maximum admissible concentration of 0.1 µg/L for any individual product, or 0.5 µg/L for total pesticides. In the mid-1990s, groundwater samples with residues above 0.1 µg/L ranged from about 5 per cent in Denmark to 50 per cent in Italy, Spain and the Netherlands [87]. In the US, some 9900 wells out of 68,800 tested between 1971 and 1991 had residues exceeding EPA standards for drinking water. Some products have been found long after their supposed cessation of agricultural use. The National Water-Quality Assessment (NAWQA) Program of the US Geological Survey, for example, analysed 500 sites in 19 hydrologic basins in the 1990s, and found a common presence of the organochlorine products, DDT, total chlordane, dieldrin, and total PCBs [88].

2.8 Organic Cotton

Cotton grown without the use of any synthetically compounded chemicals (i.e., pesticides, fertilizers, defoliants, etc.) is considered as ‘‘organic’’ cotton. It is produced under a system of production and processing that seeks to maintain soil fertility and the ecological environment of the crop. To be sold as organic it must be certified [29].

However, chemicals considered natural can be used in the production of organic cotton as well as natural fertilizers. The different certification organizations have similar lists for allowed chemicals. Bacillus thuringiensis (Bt), a naturally occurring soil bacterium, can be used as a natural insecticide in organic agriculture. Bt is the bacterium that produces the insect toxins that scientists use to produce genes for insect-resistant biotech cottons. However, biotech cottons, containing Bt genes, are not allowed to be used for the production of organic cotton, the general reason being that the technique is synthetic not natural. The production of cotton using organic farming techniques seeks to maintain soil fertility and to use materials and practices that enhance the ecological balance of natural systems and integrate the parts of the farming system into an ecological whole. Conventional agriculture often relies on targeted solutions e.g. the application of herbicide to resolve a weed problem. In contrast, organic production normally relies on solutions implemented at a systems level. This can be exemplified in the design of rotations for nutrient cycling and weed, pest and disease control. Conventional farming can be described as a linear approach to agriculture, while organic farming represents a network or ecological approach [89].

A three-year transitional period from conventional to organic cotton production is required for certification. Cotton produced during this three-year period is described variously as transitional, pending certification (in California), or organic B (in Australia). European Union (EU) regulations, International Federation of Organic Agriculture Movements (IFOAM) standards, and the US National Organic Standards (NOP) have helped to formulate organic farming legislation and standards throughout the world. Certifying companies develop their own standards but all are essentially comparable. Organic cotton producers have to commit to follow the standards set by the certifying organizations or companies, which includes verification through field visits by independent third parties. The certifying agency must be accredited, recognized by buyers, and the system must be independent and transparent [90].

Certified organic cotton production began in the United States and Turkey in the late 1980s and early 1990s, followed by Egypt, Uganda, India and Peru. The production of organic cotton has grown from 6 countries in the 1992/1993 crop season to 24 countries in 2006/2007 [3]. India, Syria, Turkey, China, Tanzania, United States, Uganda, Peru, Egypt and Burkina Faso are the top 10 organic cotton producing countries in order by rank. India recently overtook Turkey's long time standing as the number one producer, and now accounts for 55% of organic cotton production worldwide. The organic cotton market has grown from 2075 metric tons in 1992/1993 to 145872 metric tons in 2007 and now represents 0.2% of the global cotton production. It is hoped that this increased market demand will create a 'pull-through' effect to convert chemical intensive farming systems to organic [91].

2.9 Analytical Methods for Residual Pesticides

The introduction of second generation pest control agents, largely synthetic organics such as DDT, 2,4-D, and ethyl parathion, from the 1940s on, had invited heightened consumer concern, regulatory attention and monitoring activity. The collection of residue monitoring data, begun in the 1950s, has played a major role in understanding how residues are deposited and dissipated [92].

Major progress has been made in the development of analytical methods for pesticide residue since the early days of pesticide residue regulation. Colorimetric methods were the best methods available at that time. These methods had high limits of quantitation (LOQ), being approximately 1mg/kg. Before 1960 individual procedures were used for nearly each pesticide. As the number of pesticides increased, the application of a large number of individual methods to determine them became economically impracticable. The physical and chemical properties of pesticides may differ considerably. There are several acidic pesticides; others are neutral or basic. A number of compounds are very volatile, but several do not evaporate at all. Without question, the most efficient approach to pesticide analysis involves the use of multiclass, multiresidue methods (MRMs). MRMs are preferable because many pesticides can be determined in a single analysis, reducing time and costs. The basic units of pesticide residue analysis are:

(1) Sampling (2) Extraction of pesticides from samples (3) Clean-up (4) Identification and quantitative determination of pesticide residue [93].

Colorimetry, polarography, and both paper and thin-layer chromatography provided minimum analyte detectabilities of 10^{-5}–10^{-8}g (10μg–10ng). GC with element-selective detectors or electron capture detection (ECD) provided analyte detection limits of 10^{-9}–10^{-12}g (1ng–1pg). Hyphenated techniques, such as gas chromatography/mass spectrometry (GC/MS), gas chromatography/tandem mass spectrometry (GC/MS/MS) and high-performance liquid chromatography/mass spectrometry (HPLC/MS) also gave analyte detectabilities of 10^{-9}-10^{-12} g, but with exceptional, often single analyte selectivity. Enzyme-linked immunosorbent assay (ELISA) and other antibody based immunoassays operate in the same range, often at significantly reduced costs. When pushed to the limit by overriding human health concerns, residue chemists have achieved detection limits of 1ppt ($1ngkg^{-1}$) or even into the low ppqr ($1pgkg^{-1}$) range [92].

Time-of-flight (TOF) instruments have recently gained popularity in quantitative analysis. TOF mass spectrometers (TOF-MS) have advantages over quadrupole MS spectrometers because of their fast acquisition rates and high resolution capabilities. Gas chromatograms have narrow peaks and require a fast-scanning detector. TOF-MS, unlike scanning instruments, have the ability to acquire chromatograms in micro seconds, depending on the acceleration potential. Normally, TOF-MS have been used for accurate mass measurements for empirical formula verification. However, over the past decade, they have been used quantitatively as well. Cajka and Hajslova [94] demonstrated the high-resolution TOF-MS as a powerful tool for reliable detection and accurate quantitation of pesticide residue even at very low concentration levels.

Traditional solvent extraction remains the most widely used method for sample preparation with a tendency to suppress the clean-up procedures of this isolation step [93]. However, miniaturization has become a dominant trend in the analysis of low-level contaminants in food and environmental samples. This has resulted in a significant reduction in the volume of hazardous and expensive solvents. Typical examples of miniaturization in sample preparation techniques are micro liquid/liquid extractions (in-vial) and solvent-free techniques such as solid-phase micro extraction (SPME). Combined with state-of-the-art analytical instrumentation, this trend has resulted in faster analyses; higher sample throughputs and lower solvent consumption, whilst maintaining or even increasing assay sensitivity [95].

GC coupled with EI-MS and LC with MS-MS using ESI is the most important detection techniques in pesticide residue analysis today. Alder et al. [6] compared the scope and sensitivity of GC coupled with EI and single quadrupole MS with LC combined with tandem mass spectrometry for the analysis of 500 high-priority pesticides concluding that both techniques are still needed to cover the wide range of pesticides to be monitored.

Table 9: Capabilities of the Different Analyzers for Pesticide Residue Analysis [96]

Analyzer	Advantages	Disadvantages
Quadrupole (Q): GC-MS, LC-MS	High sensitivity in SIM mode (0.1–1 pg), good dynamic range (five orders of magnitude), good selectivity in CI, low cost	Poor sensitivity in scan mode (50 –500 pg), low selectivity for complex matrices, SIM needs pre selection, unit mass resolution
Quadrupole ion trap (QIT): GC-MS, GC-MS/MS, LC-MS, LC-MS/MS	High/medium sensitivity in scan and product ion scan modes (0.1 –10 pg), library-searchable EI and product ion spectra, good selectivity in CI, MS^n, fast acquisition rate, low cost	Low selectivity for complex matrices in MS mode, limit on number of ions that can be determined simultaneously, limited dynamic range (3–4 orders of magnitude), limited mass range in MS/MS, unit mass resolution
Triple Quadrupole (QqQ): GC-MS/MS, LC-MS/MS	Excellent sensitivity (10 –100 fg) and selectivity in MRM mode, good dynamic range (five orders of magnitude), concurrent monitoring of many channels	The number of MRM channels that can be monitored at any one time is limited, MRM needs pre selection, unit mass resolution, high cost
High-speed time-of-flight (TOF): GC-MS	High sensitivity (0.1 –1 pg), library-searchable EI spectra, very fast acquisition rate	Low selectivity, limited dynamic range (four orders of magnitude), unit mass resolution, high cost
Enhanced resolution TOF: GC-MS, LC-MS	High sensitivity (0.1 –1 pg), good selectivity, accurate mass, fast acquisition rate	Limited dynamic range (four orders of magnitude), not true ‘‘ high resolution,’’ high cost
Qq-linear IT (QqLIT): LC-MS/MS	Excellent sensitivity (10 –100 fg) and selectivity in MRM mode, high sensitivity in product ion scan mode, MS^n	Unit mass resolution, high cost
QTOF: LC-MS/MS	High sensitivity (0.1 –1 pg), good selectivity, accurate mass of both precursor and product ions, fast acquisition rate	Limited dynamic range (four orders of magnitude), not true high resolution, high cost

Moreover, important advances were made in the development and application of GC combined with QqQ mass spectrometer. The detection of pesticides by GC-tandem quadrupole mass spectrometry is now supplanting current GC-MS detection with a single quadrupole [93]. The use of alternative mass analyzers to the single quadrupole (Q) (i.e., various types of ion trap, triple quadrupoles, and time-of - flight) and their various combinations (e.g., QTOF) has improved the capabilities of the instruments available.

Table 9 provides an overview of the advantages and disadvantages of each analyzer for both GC-MS and LC-MS. The vast majority of pesticides sought is amenable to multiresidue approaches and can now be thoroughly isolated from water and complex

food matrices without the large amounts of natural material coextracted with the pesticides interfering with the analysis. For example, out of ~ 400 pesticides routinely targeted using the QuEChERS method, 217 are analyzed employing LC-M S-MS and 187 employing GC-MS and GC-TOF MS techniques [96].

The improvement in detection limits (and in accuracy and precision) can be ascribed to the following advances in techniques and instrumentation:

1. Advent of commercial ultraviolet (UV) visible spectrophotometers, beginning with the Beckman DU spectrophotometer, and associated derivatization techniques to form UV-absorbing or colored derivatives;

2. Development of chromatography, with its unsurpassed ability to resolve individual chemical species;

3. Development of class- and chemical-specific spray reagents (paper and thin-layer) and electronic detectors for GC and high-performance liquid chromatography (HPLC), using element-selective and electron capture (GC), UV visible (HPLC), and mass spectrometry (both GC and HPLC).

These high-profile developments were accompanied by improvements in technology such as electronics, particularly the advent of transistors and integrated circuit boards, fiber optics, and computer interfaces.

There are a number of international agencies and governmental organizations operating with expertise in pesticide residue analysis. These include the ISO (International Organization for Standardization), AOACI (Association of Official Analytical Chemists International), IUPAC (International Union of Pure and Applied Chemistry), Codex Alimentarius, OECD (Organization for economic Cooperation and Development), and FAO/WHO (Food and Agriculture Organization of the United Nations, World Health Organization). These organizations have initiatives to standardize methods and follow established protocols for producing acceptable data [92]. Residue analytical methods are needed to enforce the legally based limits or guidance values and to perform monitoring projects [97].

Analyses must prove reliable, be capable of residue measurement at very low levels (sub ppb), and also provide unambiguous evidence of the identity and magnitude of any

residues detected. More recently, additional emphasis has been on shortening analysis times to deal with high sample throughput [96].

2.9.1 Pesticide Determination by Electrochemical Biosensors

The techniques of gas chromatography, liquid chromatography and thin film chromatography coupled with different detectors and the different types of spectroscopy are the most commonly used methods for the recognition of residual pesticides [98]. However, these techniques, which are time consuming, expensive and require highly trained personnel, are available only in sophisticated laboratories [99]. Biosensors are increasingly becoming powerful tools in clinical diagnostics, drug detection, and food and environmental monitoring. This is the reason behind the great amount of research focused on new materials and immobilization strategies in these electro analytical devices [100]. The potential growth in the world biosensor industry is remarkable; the emerging Biosensor market is expected to grow at over 9% in the coming years thus becoming one of the fastest growing sectors in the World. As stated by the recent report published by Global Industry Analysts Inc, United States and Europe dominate the global market for medical biosensors, collectively capturing 69.73% share estimated in 2008. The market in Asia-Pacific is projected to reach US$794 million by the year 2012 [101].

The introduction of Biosensors was based on the Clark oxygen electrode and these are characterized by the direct spatial combination of a matrix-bound biologically active substance (receptor) with an electronic device [102].

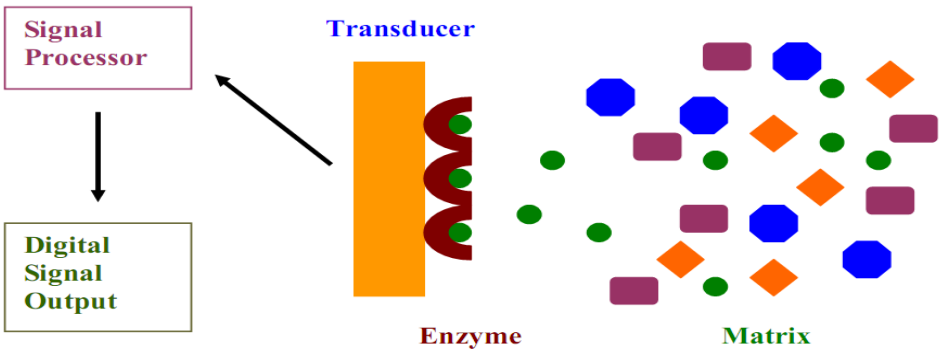


Figure 16: Schematic diagram showing the functioning of a biosensor device [104]

Biosensors unify the advantages of semiconductor technology with the specificity of recognition provided by biological materials and processes. A biosensor is an analytical device incorporating an intimate combination of a biological sensing element (for

recognizing a specific biomolecule through a reaction, adsorption or physico-chemical process) with a traditional physical or chemical transducer (for conversion of the recognition into a useable signal) [103] as shown in Figure 16.

The following sequence of processes takes place in biosensors:
(I) Specific recognition of the analyte;
(II) Transduction of the physiochemical effect caused by the interaction with the receptor into an electrical signal;
(III) Signal processing and amplification.

In operation the biosensor measures the change in the concentration of a co-reactant that reacts with the analyte or a co-product, which is produced with the analyte of a biological reaction (e.g. enzyme reaction). When an electrode is used as a transducer in a biosensor, the electrode converts the change in concentration of a product of a biological reaction into an electrical signal [104].
Biosensors use biologically-derived components integrated with a suitable transducer. Due to the development and implementation of biosensors has been narrowly related to the sensors technology advances. A general scheme of a biosensor device is shown in Figure 17. Biosensors are usually classified into various basic groups according either to the method of signal transduction, or to the bio recognition principle.

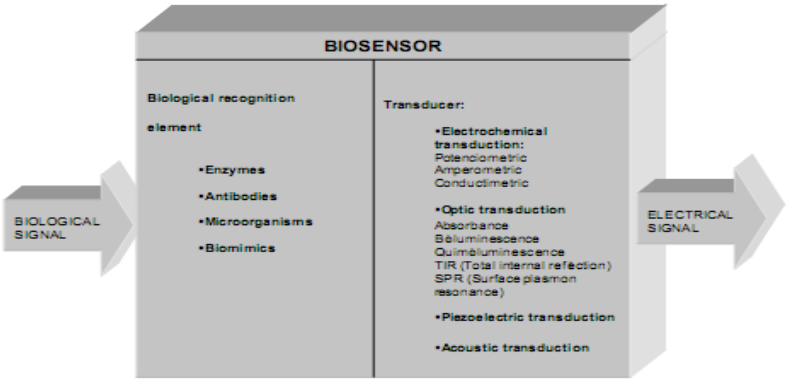


Figure 17: A general scheme of a biosensor device [105]

The biological recognition element can be enzymes, antibodies, whole cells including microbial, plant and animal cells, sub-cellular organelles, tissue slices, several plant glycoproteins that act like antibodies, and lately bio mimics (synthetic molecules with similar affinity to biological ones). These biological recognition systems have been linked to electrochemical, optical-electronic, optical, and acoustic transducers [105].

2.9.1.1 Cholinergic Neurotransmission & AChE Inhibition

Biosensors based on the inhibition of Acetylcholinesterase (AChE) have been widely used for the detection of OP compounds [7]. The organophosphates and carbamates are powerful inhibitors of AChE [8]. Acetylcholinesterase (AChE; EC 3.1.1.7.) is an enzyme participating in cholinergic neurotransmission [106]. The toxicity or mode of action of OP compounds can be attributed to the inhibition of the enzyme, AChE. AChE is a globular protein and its three-dimensional structure is known. Its physiological substrate is acetylcholine (ACh). The active site of AChE consists of two subsites, anionic and esteratic sites. The anionic site is represented by a glutamate ion. The esteratic site has serine moiety and histidine as well as tyrosine residues. This enzyme is essential for the central nervous system, and being present in both humans and insects. The normal function of AChE is the hydrolysis of acetylcholine neurotransmitter in the synaptic membrane to prevent its accumulation, and as a result forming acetylated enzyme and releasing choline.

$$\text{Choline} + \text{Acetyl Coenzyme A} \overset{\text{ChAT}}{\Longleftrightarrow} \text{Acetylcholine} + \text{Coenzyme A}$$

$$\text{Acetylcholine} \overset{\text{AChE}}{\longrightarrow} \text{Acetate} + \text{Choline}$$

ACh serves as a transmitter at synapses in the ganglia of the visceral motor system, and at a variety of sites within the central nervous system. The high percentage of released choline is transported back into the nerve ending for reconversion to acetylcholine and storage (Fig 18).

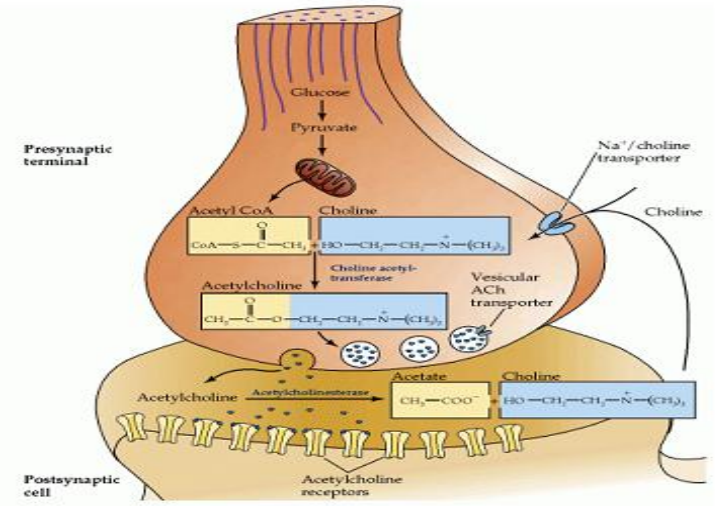


Figure 18: Acetylcholine metabolism in cholinergic nerve terminals [109]

This degradation process results in a lowered level of acetylcholine. OP compounds covalently block the active site of serine residue of AChE by undergoing nucleophilic attack to produce a serine-phosphoester adduct. This irreversible inactivation leads to an excess accumulation of acetylcholine in the peripheral and central nervous system causing cholinergic manifestations. At high doses, there is depression of the respiratory centre in the brain, followed by peripheral neuromuscular blocked causing respiratory paralysis and death [107, 108].

2.9.1.2 Enzyme Inhibition Mechanism

Enzymes, the catalysts of biological systems, are remarkable molecular devices that determine the patterns of chemical transformations. They also mediate the transformation of one form of energy into another. The most striking characteristics of enzymes are their catalytic power and specificity. Enzymes are highly specific both in the reactions that they catalyze and in their choice of reactants, which are called substrates. An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions. Catalysis takes place at a particular site on the enzyme called the active site (Fig 19). Enzymes accelerate reactions by factors of as much as a million or more. Enzymes accelerate the attainment of equilibria but do not shift their positions. The equilibrium position is a function only of the free-energy difference between reactants and products [110].

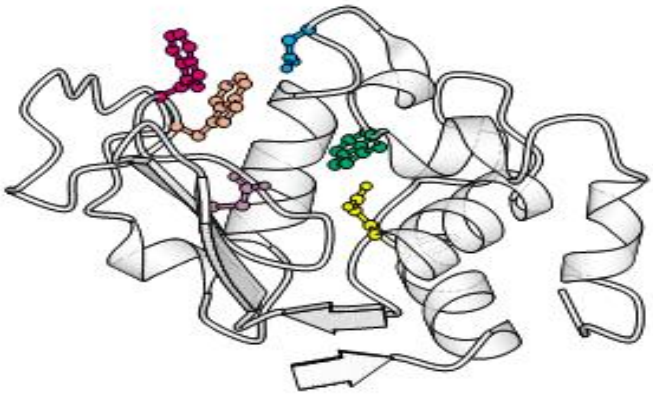


Figure 19: Ribbon diagram of the enzyme lysozyme with several components of the active site shown in color [110]

Enzymes are classified into six major groups on the basis of the type of reaction that they catalyze. Each enzyme has a unique four-digit classification number. Virtually all enzymes are proteins, although some catalytically active RNAs have been identified.

The active site is the region of the enzyme that binds the substrate, to form an enzyme–substrate complex, and transforms it into product. The active site is a three-dimensional entity, often a cleft or crevice on the surface of the protein, in which the substrate is bound by multiple weak interactions. Two models have been proposed to explain how an enzyme binds its substrate: the lock-and-key model and the induced-fit model [111].

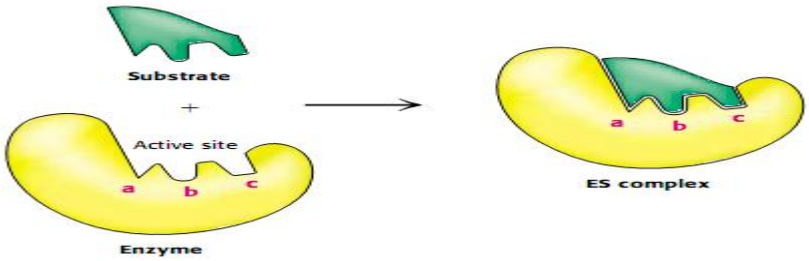


Figure 20: Lock-and-key model of enzyme–substrate binding [110]

The specificity of binding depends on the precisely defined arrangement of atoms in an active site. Because the enzyme and the substrate interact by means of short-range forces that require close contact, a substrate must have a matching shape to fit into the site. Emil Fischer's analogy of the lock and key (Fig 20), expressed in 1890, has proved to be highly stimulating and fruitful. However, we now know that enzymes are flexible and that the shapes of the active sites can be markedly modified by the binding of substrate, as was postulated by Daniel E. Koshland, Jr., in 1958. The active site of some enzymes assumes a shape that is complementary to that of the transition state only after the substrate is bound. This process of dynamic recognition is called induced fit (Fig 21) [110].

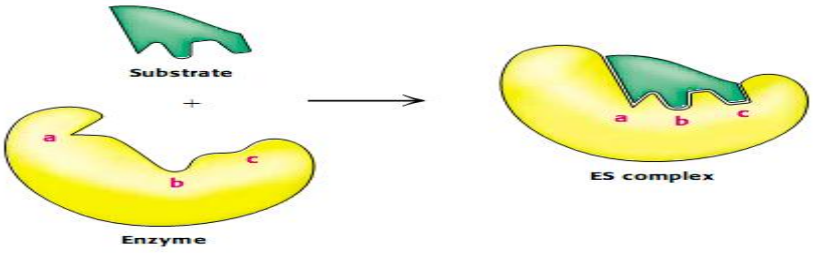


Figure 21: Induced-fit model of enzyme–substrate binding [110]

Enzyme reactions are usually formulated as simple processes, e.g., for the case of a single substrate reaction:

$$\mathrm{E} + \mathrm{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}\,.$$

Where the rate constants k_1, $k_{-1}$and k_2 describe the rates associated with each step of the catalytic process. At low [A], V_o is directly proportional to [A], while at high [A] the velocity tends towards a maximum velocity (V_{max}).

The Michaelis-Menten equation:

$$V_O = \frac{V_{max} \cdot [A]}{K_M + [A]}$$

describes these observations and predicts a hyperbolic curve of V_0 against [A]. The Michaelis constant, K_M, is equal to the sum of the rates of breakdown of the enzyme–substrate complex over its rate of formation, and is a measure of the affinity of an enzyme for its substrate. *Vmax* and K_M can be determined experimentally by measuring *Vo* at different substrate concentrations, and then plotting $1/V_0$ against 1/[A] in a double reciprocal or Lineweaver–Burk plot. The intercept on the *y*-axis is equal to 1/*Vmax*, the intercept on the *x*-axis is equal to $-1/K_M$ and the slope of the line is equal to K_M /*Vmax* [111].

Vmax, is the reaction rate when the enzyme is fully saturated with substrate and K_M, the Michaelis constant, is the substrate concentration at which the reaction rate is half maximal. The maximal rate, *Vmax*, is equal to the product of k_2 or *kcat* and the total concentration of enzyme. The kinetic constant *kcat*, called the turnover number, is the number of substrate molecules converted into product per unit time at a single catalytic site when the enzyme is fully saturated with substrate. Turnover numbers for most enzymes are between 1 and 10^4 per second. The ratio of *kcat* /K_M provides a penetrating probe into enzyme efficiency [110].

On closer scrutiny, however, such mechanisms prove much more complex, a process composed of several partial steps:

$$\mathrm{E} + \mathrm{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E^*A} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E^*P} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{EP} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}\,.$$

In a rapid equilibrium, an initial loose association complex is formed between enzyme E and substrate A. Subsequently, the enzyme shifts into its active form E* and can then convert substrate into product P. Upon reversion of the enzyme into its original state E, the product molecule dissociates, and the enzyme is ready to interact with another substrate molecule. The complete mechanism consists of a sequence of five partial reactions. For a full extensive characterisation, five equilibrium constants, or ten rate

constants, respectively, have to be determined. Enzyme mechanisms become even more complicated when involving two or more substrates, cofactors, and effectors [112].

The activity of many enzymes can be inhibited by the binding of specific small molecules and ions [110]. This means of inhibiting enzyme activity serves as a major control mechanism in biological systems. Many inhibitors exist, including normal body metabolites, foreign drugs and toxins [111].

Enzyme inhibition can be either reversible or irreversible. An irreversible inhibitor dissociates very slowly from its target enzyme because it has become tightly bound to the enzyme, either covalently or non-covalently. Some irreversible inhibitors are important drugs. Penicillin acts by covalently modifying the enzyme transpeptidase, thereby preventing the synthesis of bacterial cell walls and thus killing the bacteria. Aspirin acts by covalently modifying the enzyme cyclooxygenase, reducing the synthesis of inflammatory signals [110].

Irreversible binding of inhibitor to an enzyme can be described by the following reaction scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI_i$$

The inhibitor initially forms a non-covalent association complex EI with the enzyme, which will be transformed by an irreversible process into the inactive complex EI_i [112]. A competitive inhibitor typically has close structural similarities to the normal substrate for the enzyme. Thus it competes with substrate molecules to bind to the active site (Fig 22a). The enzyme may bind either a substrate molecule or an inhibitor molecule, but not both at the same time (Fig 22b).

The competitive inhibitor binds reversibly to the active site. At high substrate concentrations the action of a competitive inhibitor is overcome because a sufficiently high substrate concentration will successfully compete out the inhibitor molecule in binding to the active site. Thus there is no change in the *Vmax* of the enzyme but the apparent affinity of the enzyme for its substrate decreases in the presence of the competitive inhibitor, and hence K_M increases.

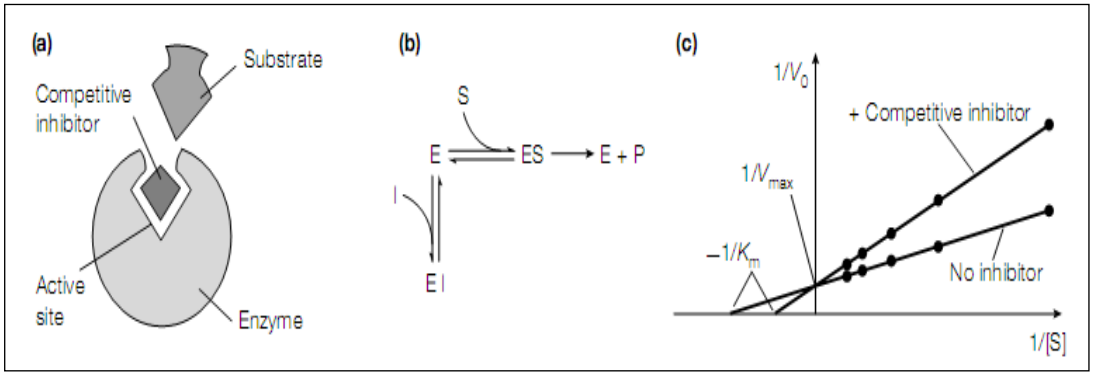


Figure 22: The characteristics of competitive inhibition. (a) A competitive inhibitor competes with the substrate for binding at the active site; (b) the enzyme can bind either substrate or the competitive inhibitor but not both; (c) Lineweaver– Burk plot showing the effect of a competitive inhibitor on K_M and *Vmax* [110]

A non-competitive inhibitor binds reversibly at a site other than the active site (Fig 23a) and causes a change in the overall three-dimensional shape of the enzyme that leads to a decrease in catalytic activity. Since the inhibitor binds at a different site to the substrate, the enzyme may bind the inhibitor, the substrate or both the inhibitor and substrate together (Fig 23b).

The effects of a non-competitive inhibitor cannot be overcome by increasing the substrate concentration, so there is a decrease in *Vmax*. In non competitive inhibition the affinity of the enzyme for the substrate is unchanged and so K_M remains the same. Non competitive inhibition can be recognized on a Lineweaver–Burk plot, since it increases the slope of the experimental line, and alters the intercept on the *y*-axis (since *Vmax* is decreased), but leaves the intercept on the *x*-axis unchanged (since K_M remains constant; Fig 23c) [111].

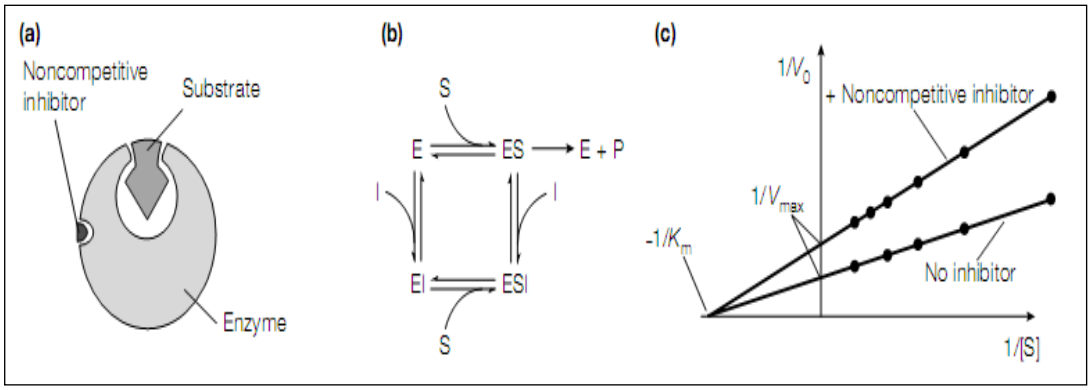
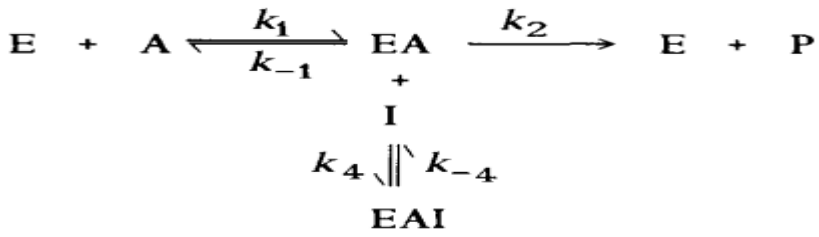


Figure 23: The characteristics of noncompetitive inhibition. (a) A noncompetitive inhibitor binds at a site distinct from the active site; (b) the enzyme can bind either substrate or the noncompetitive inhibitor or both; (c) Lineweaver–Burk plot showing the effect of a noncompetitive inhibitor on K_M and *Vmax* [110]

Uncompetitive Inhibition binds the inhibitor exclusively to the enzyme-substrate complex. Such a mechanism will be realized when the binding site for the inhibitor is only formed in interaction with substrate [112].



A more complex pattern, called mixed inhibition, is produced when a single inhibitor both hinders the binding of substrate and decreases the turnover number of the enzyme [110].

2.9.2 AChE Inhibition based Biosensors

Enzyme-based biosensors have emerged during past few years and based on the principle of inhibition of AChE and electrochemical or optical based detection. The inhibition of AChE is measured by direct or indirect measurement of its activity [108].

Acetylthiocholine (ATCh) is used instead of the natural ACh substrate to convert the response of AChE to the electrical signal. The electro active Acetylthiocholine (ATCh) is preferred due to its better redox activity and it can both be oxidized and reduced to provide better movement of electrons in the amperometric biosensors. The product of enzymatic reaction Thiocholine (TCh) can be detected by its reaction to disulfide at the electrode at 350 mV [113].

$$ATCh + H_2O \xrightarrow{AChE} TCh + CH_3COOH$$

$$2TCh \xrightarrow{anode} disulphide + 2e^-$$

The rate of inhibition (%) is calculated before and after incubation with OP compounds as

$$I\,\% = 100 \times \frac{I_O - I_i}{I_O}$$

Where Io is current before inhibition and I_i is current after inhibition. In the development of biosensors immobilization of enzymes is the critical step in maintaining enzyme activity, stability and shelf life of electrode. Various techniques

are used such as physical entrapment, microencapsulation, covalent binding, adsorption and cross-linking.

Enzyme activity and stability on the transducer surface is governed by the procedure followed for the immobilization and chemical nature of used matrices. The adopted immobilization method should be sufficiently strong to provide good mechanical stability of biosensor, and sufficiently soft to provide optimal conformation and freedom of the enzymes, which is crucial for reaching sufficient enzymatic activity [114]. Several approaches have been followed for immobilization of enzyme on the transducer/electrode surface, such as adsorption, cross-linking with bifunctional chemical reagents, binding with dendrimer layers, entrapment in different matrices, including layer of cross-linked bovine serum albumin and electro polymerization, and more recently bioaffinity attachment using concanavalin A, etc. However, no method can be commonly used for all the enzymes by retaining their complete activity [115].

AChE was encapsulated in sol-gel film on a glass cap that could be fixed on an optical fibre [108]. Sol–gel technology provides an attractive way for the immobilization of biological entities including full cell, enzyme, protein and antibody or antigen due to the inert low temperature process [116]. Recently, the nanoparticles and carbon nanotube (CNT) have received considerable attention to increase the sensitivity of the biosensor due to their high conductivity, catalytic and electrical properties [117].

For biosensors based on direct electron transfer of protein, the absence of mediator is the main advantage, providing them with superior selectivity, both because they should operate in a potential window closer to the redox potential of the enzyme and are therefore less prone to interfering reaction, but also because of the lack of yet another reagent in the reaction sequence, which simplifies the reaction system. Another attractive feature of the system, based on direct electron transfer of protein, is the possibility of modulating the desired properties of an analytical device using protein modification with genetic or chemical engineering techniques on one hand and novel interfacial technologies on the other [118].

In another article [119] acetylcholinesterase immobilized on the surface of screen-printed electrodes by adsorption and covered with a Nafion film is reported. The incorporation of the TCNQ mediator in a carbon working electrode permits a dramatically decrease of the potential necessary for oxidation of thiocholine.

The great advantage of the inhibition based acylcholinesterase sensors for OP pesticides quantification is their sensitivity [120]. However, they are poor in selectivity and are rather slow and tedious since the analysis involves multiple steps of reaction such as measuring initial enzyme activity, incubation with inhibitor, measurement of residual activity, and regeneration and washing [115].

AChE based biosensors have poor stability in front aggressive conditions, matrix effects, and sometimes lack of reproducibility between different batches of production, however the screen-printed biosensors may be the answer to the problem [121]. Another approach is molecularly imprinted polymers (MIPs), one of the biomimetic materials lately developed, which can overcome these limitations, offering a very good specificity without limitation of molecular weight or size [105].

2.9.3 Gas Chromatography coupled to Tandem Mass Spectrometry

Gas chromatography (GC) is undoubtedly one of the key techniques used for screening / identification / quantification of many groups of non-polar and/or semi-polar food toxicants (or their GC amenable derivatisation products).

The high attainable separation power (potential number of theoretical plates) in combination with a wide range of the detectors employing various detection principles to which it can be coupled makes GC an important, often irreplaceable tool in the analysis of (ultra) trace levels of toxic food components that may occur in such complex matrices as foods and feeds.

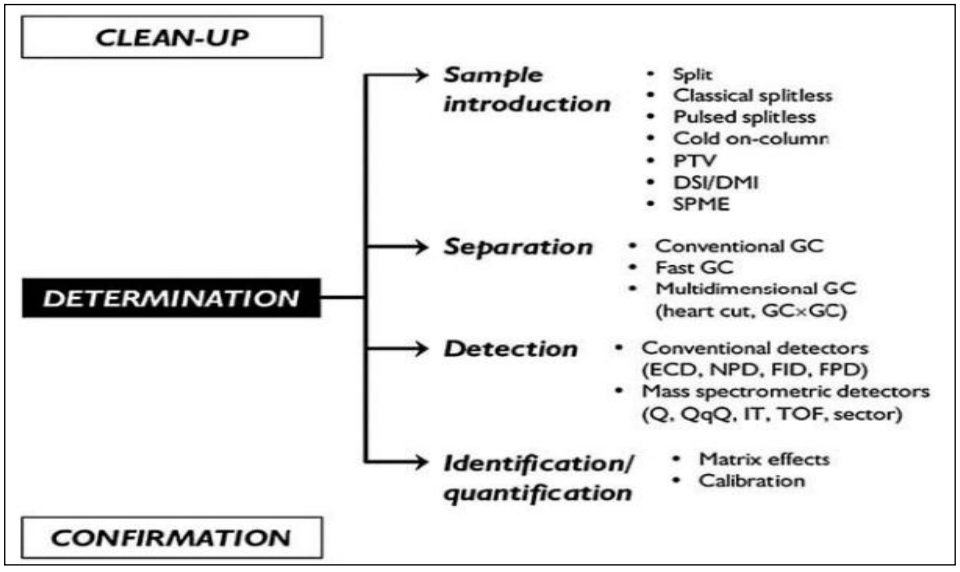


Figure 24: Basic steps typically involved in GC analysis of organic food toxicants [122]

In practice, the methods used for analyses of food toxicants typically consist of basic steps as shown in Figure 24: (i) isolation from a representative sample (extraction step); (ii) separation from bulk co-extracted matrix components (clean-up step); (iii) identification and quantification (determinative step). The last step is often optionally followed by (iv) confirmation of results.

Like a good marriage, both gas chromatography and mass spectrometry bring something to their union. GC can separate volatile and semi volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified, but it cannot readily separate them.

Gas chromatography and mass spectrometry are, in many ways, highly compatible techniques. In both techniques, the sample is in the vapor phase, and both techniques deal with about the same amount of sample (typically less than 1 ng) [123].

GC coupled with mass selective detector was needed for confirmation to avoid biased results. Advantages of GC–MS over traditional approaches include:

(*i*) Simultaneous quantification and confirmation of target analytes

(*ii*) Detection and identification of non-target sample components

(*iii*) Possibility to spectrometrically resolve co-eluting peaks

However, under certain circumstances simple MS detectors such as single quadrupole may due to high chemical noise fail to detect residues overlapped by abundant matrix interferences (this may be the case when low, unspecific ions *m/z* are yielded from an analyte) [122].

2.9.3.1 Tandem Mass Spectrometry

Tandem mass spectrometry, abbreviated MS/MS, is any general method involving at least two stages of mass analysis, either in conjunction with a dissociation process or in a chemical reaction that causes a change in the mass or charge of an ion.

In the most common tandem mass spectrometry experiment a first analyser is used to isolate a precursor ion, which then undergoes spontaneously or by some activation a fragmentation to yield product ions and neutral fragments:

$$m_p^+ \longrightarrow m_f^+ + m_n$$

A second spectrometer analyses the product ions. The principle is illustrated in Figure 25. An ion M1 is selected by the first spectrometer MS1, fragmented through collision, and the fragments are analysed by the second spectrometer, MS2. Thus ions with a selected m/z value, observed in a standard source spectrum, can be chosen and fragmented so as to obtain their product ion spectrum.

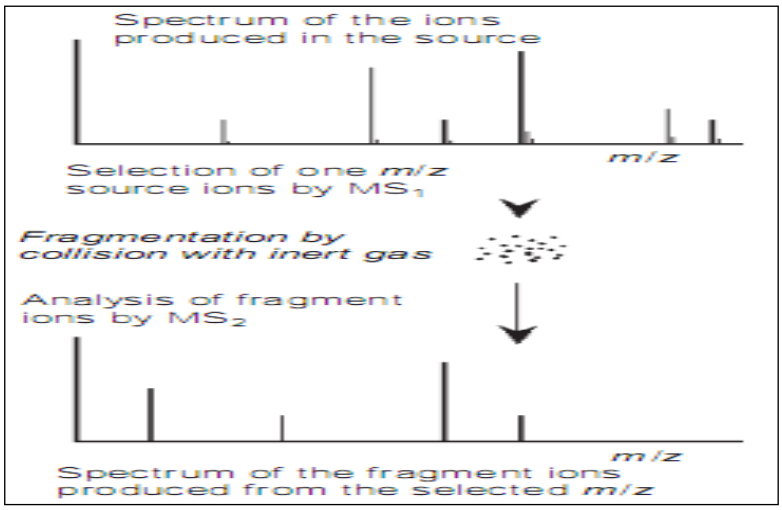


Figure 25: Principle of MS/MS [124]

The four main scan modes available using tandem mass spectrometry are represented in Figure 26. Many other MS/MS scan modes are also possible. MS/MS methods generally involve activation of selected ions, typically by collision with an inert gas, sufficient to induce fragmentation (CID). CID stands for collision-induced dissociation, as occurs when an inert gas is present in the collision cell [124].

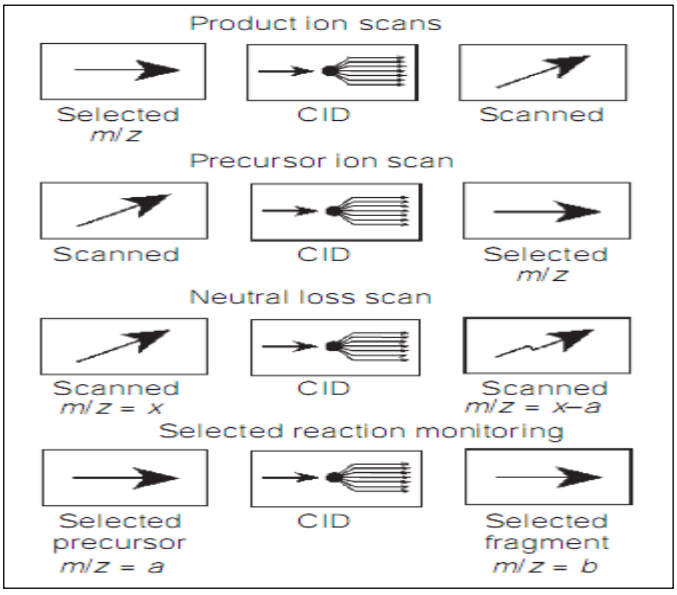


Figure 26: Main processes in tandem mass spectrometry (MS/MS) [124]

The main advantage of using MS/MS is the discrimination against the chemical noise, which can originate from different sources (e.g. matrix compounds, column bleed and contamination from an ion source) [122]. Figure 27 illustrates the effect of multiple MS/MS steps on S/N ratios. Each MS-step leads to some loss in intensity of the analyte signal, but a greater loss in the noise signal and, consequently, S/N increases [125].

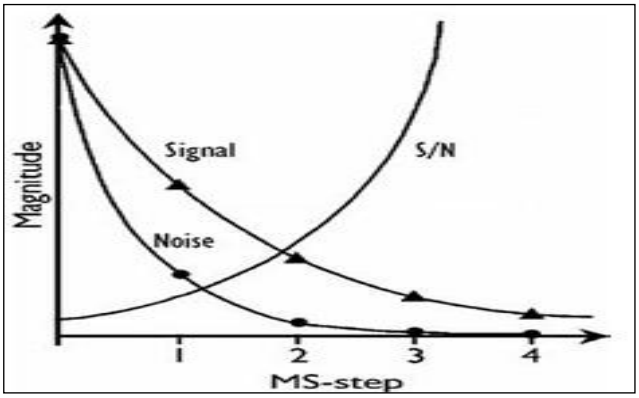


Figure 27: Influence of MS-step *vs.* signal, noise, and S/N (adapted) from [125]

The increase in the use of GC-MS-MS for pesticide residue analysis provides an increase in selectivity and hence a more secure confirmation of identity [96].

GC-MS has a few limitations. Only compounds with vapor pressures exceeding about 10^{-10} torr can be analyzed by GC-MS. Many compounds that have lower pressures can be analyzed if they are chemically derivatized (for example, as trimethylsilyl ethers). Determining positional substitution on aromatic rings is often difficult. Certain isomeric compounds cannot be distinguished by mass spectrometry (for example, naphthalene versus azulene), but they can often be separated chromatographically [123].

Three important molecular properties that determine if the pesticide will or will not be recovered and detected are polarity, volatility, and thermal lability. Generally, polarity for non-ionic pesticides ranges from the lipophilic OCs (e.g., p,p′-DDE) and synthetic pyrethroids (e.g., deltamethrin) to the very polar , water-soluble OPs, methamidophos , and acephate. Thus, a measure of the usage of a comprehensive multiclass MRM is whether it can recover both the nonpolar and polar pesticides.

Volatility and thermal lability are important because they determine whether the pesticide can be determined by GC or not. Many pesticides are thermally labile, and will degrade in a GC due to the heated conditions of the injector and the increasing

temperature gradients applied to the column. Other separation methods, mainly high-performance liquid chromatography (HPLC), must then be used [126].

Gas chromatographic mass spectrometry is the single most important tool for the identification and quantitation of volatile and semi volatile organic compounds in complex mixtures. As such, it is very useful for the determination of molecular weights and (sometimes) the elemental compositions of unknown organic compounds in complex mixtures. Among other applications, GC-MS is widely used for the quantitation of pollutants in drinking and wastewater. It is the basis of official EPA methods. It is also used for the quantitation of drugs and their metabolites in blood and urine. GC-MS can be used for the identification of unknown organic compounds both by matching spectra with reference spectra and by a priori spectral interpretation [123].

Chapter 3: Materials and Methodology

3.1 Materials

3.1.1 Sample Collection

The samples of three different varities of cotton namely, Egyptian cotton Giza 86 (G86), Pakistani cotton MNH 93 and Indian Cotton were collected from the cultivation season 2011/2012. Both varieties have classical conventional cotton and organic cotton. For easily understandable we abbreviate the samples as follows:

Egyptian Giza Conventional Cotton	GC
Egyptian Giza Organic Cotton	GO
Pakistani Conventional Cotton	PC
Pakistani Organic Cotton	PO
Indian Conventional Cotton	IC
Indian Organic Cotton	IO

Another three cotton samples were taken after the first harvest from BahawalPur (Pakistan). The detail of these samples is as follows:

BT – 114	SH – 1	Z - 33

These samples were collected from the cultivation season 2012-2013 and the analyses were made within three months of their collection from the field.

3.1.2 Chemicals and Reagents

All the chemicals and reagents utilized were obtained commercially. Acetylcholinesterase (electric eel) (EC 3.1.1.7, 827 IU/mg), Acetylthiocholine chloride (A5626), Neostigmine methyl sulphate (N2126) and MOPSO Sodium Salt (M8767) were purchased from Sigma Aldrich and Phosphate buffer was purchased from Fluka. Tetrabutyl ammonium hydrogen sulfate and Tween (phase transfer catalysts) were purchased from Sigma Aldrich.

Following HPLC grade solvents for residual analysis have been purchased from Verkon. Selection has been made on the basis of different characteristics of each solvent. Table 10 shows the properties of the solvents used, taken from [127].

Table 10: Description of the solvents used

Solvents	**Formula**	**Density (g/mL)**	**BP (^{O}C)**	**E_T [a]**	**μ. 10^{30}/cm [b]**	**E_T^N [c]**	**Water solub. (g/100g)**	**Polar ?**	**[d]**
Methanol	CH_3OH	0.791	64.5	32.66	5.9	0.762	Miscible	Y	Green
Acetonitrile	CH_3CN	0.786	81.6	35.94	13.0	0.460	Miscible	Y	Orange
Acetone	$CH_3O\ CH_3$	0.786	56.1	20.56	9.0	0.355	Miscible	Y	Green
Dichloromethane	CH_2Cl_2	1.326	39.6	8.93	3.8	0.309	1.32	N	Red
Toluene	$C_6H_5CH_3$	0.867	110.6	2.38	1.0	0.099	0.05	N	Orange
Hexane	$CH_3(CH_2)_4CH_3$	0.659	68.7	1.88	0.0	0.009	0.014	N	Red

[a] E_T, Relative permittivity (‘‘dielectric constant’’) of the pure liquid at 250C

[b] Dipole moment in Coulombmetre (Cm), measured in benzene, tetrachloromethane, 1,4-dioxane, or n-hexane at 20-300C. 1 Debye = 3.336.10-30 Cm.

[c] ETN = derived from the transition energy at 250C of the long-wavelength visible absorption of a standard pyridinium N -phenolate betaine dye

[d] The Pfizer ‘‘traffic light’’ solvent preference system [128]

3.1.3 Pesticide Standards

Different Pesticide standards were purchased commercially and their purity certified by the supplier to be greater than 99%. Individual pesticides like Spirotetramet and Imidacloprid were purchased from Absolute Standards, USA. Pesticide Mix 155 and Pesticide Mix 17 were purchased from Dr. Ehrenstorfer GmbH, Germany. The detail of Pesticide Mix 155 is descibed in detail alongwith their class and their toxicity ranking in Appendix 1. Mix 155 abbreviated as KF contains 18 different pesticides mostly of which are the organophosphorous (OP) and carbamates.

The class and the degree of toxicity recommended by World Health Organization (WHO) and Environmental Protection Agency (EPA) is also mentioned for each individual compound. The data is taken from Pesticide Action Network (PAN) Pesticide Database [129].

The description of Mix 18 is shown in Appendix 5 which contains 16 different pesticides, mostly of which are organochlorine (OC) pesticides. Flubendiamide, Pesticide Mix 3, Pesticide Mix 14 and Pesticide Mix 18 were purchased from AccuStandard, USA. The compounds of Pesticide Mix 3 are shown in Appendix 3 and Appendix 4 describes the detial of compounds in the Mix 17 which contain all OC compounds. The detail of Mix 14 can be observed in Appendix 2 which contains mostly pyrethroids.

3.2 Sample Preparation

Sample preparation is often the bottleneck in a measurement process and it is important in all aspects of chemical, biological, materials, and surface analysis. Notable among recent developments are faster, greener extraction methods and micro extraction techniques [130].

Real samples contain several interfering compounds along with the target analyte. The most common ways to reduce matrix effects are (a) selective extraction ("cleanup") and (b) dilution to bring the interfering substances below a concentration [131]. Most sample preparation procedures for GC and HPLC determination follow the basic steps like homogenization, extraction, cleanup, elution and filtration to get the final solution containing the pesticides which can be introduced into the GC or HPLC [132].

The determination of pesticides in samples at low concentrations is always a challenge. The main aim of any extraction process is the isolation of analytes of interest from the selected sample by using an appropriate extracting phase. The development of an appropriate sample preparation procedure involving extraction, enrichment, and cleanup steps becomes mandatory to obtain a final extract concentrated on target analytes. It is always necessary to carry out some pre treatments to get a homogeneous and representative subsample [5].

3.2.1 Cryogenic Homogenization

It is necessary for the determination of the residual pesticides in cotton samples to turn the sample into finely chopped or ground powder. This grinding procedure should be done carefully to avoid heat generation [133]. CryoMill was used for the homogenization with 1 cm ball.

(a) (b) (c)

Figure 28: (a) Raw cotton (b) CryoMill (c) Homogenized sample

All samples of cotton were arranged around the inside of a pre-chilled Teflon mill in the form of pallets which contained a concentric Teflon ring and Teflon puck in liquid nitrogen surrounding. Each sample was milled with two cycles. Each cycle consists of exactly two minutes for grinding with an interval of 15 seconds for cooling. After the milling the resulting powder was sampled. The raw cotton samples which had been transferred to a clean Teflon bag and sealed were held overnight in liquid nitrogen vapor. Only 2 - 3 gm of the raw cotton would fit into the mill at a time and grind successfully. After each successful homogenization, the resulting powder was pooled in another clean Teflon bag. Figure 28 shows the different steps for the cryogenic homogenization. Once the entire sample was homogenized and blended, the powder was sampled, cleaned and stored for analysis.

3.2.2 Ultra Sound Assisted Extraction (USE)

Ultrasound has been used in different operations in chemical engineering, such as waste-water treatment, drying, sonochemistry, and extraction. Ultrasonic waves are elastic waves that have a frequency above the threshold of human hearing, approximately 20 kHz. The effect of the sound waves in matter is the expansion and compression cycles. The expansion can create bubbles in a liquid and produce negative pressure that can reach a high local pressure of up to 50 MPa, intense heating with hot spots around 5000 K, and lifetimes of a few microseconds, whereas the collapse of the bubbles formed can cause cavitation. At constant ultrasound intensity, dynamic equilibrium is established between the forming and the collapsing bubbles. The collapse of cavitation bubbles near cell walls produces cell disruption. As a result, there is an enhanced solvent penetration into the cells and an intensification of the mass transfer [134].

Figure 29: Ultra sonic Extraction and Cotton samples

Ultrasound radiation provokes molecules vibration and eases the diffusion of the solvent to the sample, favoring the contact between both phases. Thanks to this

improvement, both the time and the amount of solvents are considerably reduced [135]. Ultra sound extraction method was used for the extraction from all of the cotton samples. A total of 0.5 gm homogenized sample was transferred to the flask along with 10 ml of the solvent used. The flask was placed in the extraction apparatus Sonorex at a controlled temperature of 60 ^{O}C (Fig 29). Samples were extracted for 30 minutes. The extracts were then filtered and stored for further analysis.

3.3 Experimental Methods of Investigation

Following three different techniques have been employed for the detection of residual pesticides on cotton samples.

3.3.1 Biosensor based detection

Biosensor toxicity analyzer (BTA) has been used for monitoring the activity of the inhibition of AChE with the help of sensors which is equipped with an enzymatic membrane of AChE enzyme which is immobilized.

It consists of two major parts, one of which is the Micro flow unit and the other is Bioanalyzer. The micro flow unit has the capillary arrangement which allows precise and constant flow of the liquid onto the active surface of the AChE sensor for a high level of repeatability and sensitivity in the measurements. The module has an integrated chamber in which the sensor can easily be placed or replaced [136].

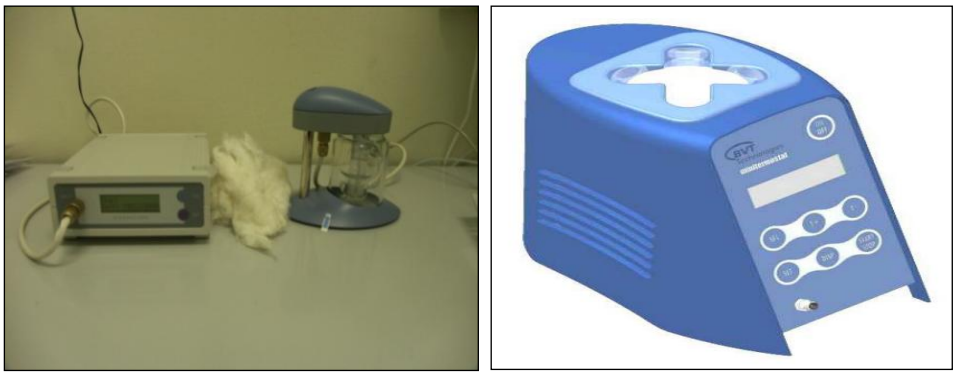


Figure 30: Biosensor toxicity analyzer (BTA) & Minithermostat device (MT-1)

Figure 30 Shows the Biosensor Toxicity Analyzer (LHS) and Minithermostat device MT-1 (RHS), a modified version of BTA. To follow the George L. Ellman's methodology, following two equipments were utilized (Fig 31). Multi function syringe pump TECHNIC I Linear Pump was used which is responsible for a continuous flow of buffer and also carry the injected analytes on to the surface of biosensor.

Figure 31: Technic I linear pump & Low pressure dose valve

The flow rate (mL/min) can be adjusted according to the needs. Low pressure dose valve v-7 from Pharmacia Biotech was used for the injection of analytes. The maximum capacity of injection per analyte was 25 µL.

3.3.1.1 Biosensor preparation

AC1.W2.RS/AChE Sensors were used for the monitoring of AChE inhibition, provided by Bvt Technologies (Fig 32). This is a thick film sensor, printed on a base made from Alumina Ceramic ($A2O_3$ -96%). This type of electrode has as a working surface 100 % platinum, as a reference 60/40 % Ag/AgCl and as auxiliary a 100 % platinum.

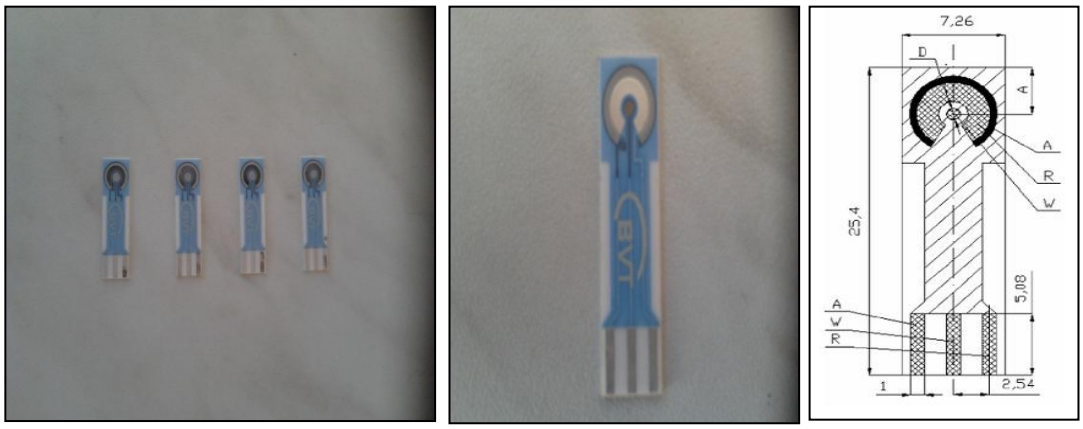


Figure 32: AC1.W2.RS Sensors from BVT Technologies

The connection of the sensor occurs through silver conducting paths which are able to minimise the potential difference between the working electrode and the potential actually applied on the system. They have a mass of 0.4 g, a width of 7.26 mm, length of 25.4 mm, and a thickness of 0.63 mm. On the surface of the working electrode is an enzymatic membrane containing 1 IU (Unit) of AChE enzyme, which is immobilized. The diameter of the immobilized bioactive membrane is 2 mm and the mean applied activity is 1 unit/mm^2. AC1.W2.R1 sensors without immobilization of AChE were used for the enzymatic inhibition optimization.

3.3.2 Life cycle assessment of single cell Algae

Algae Growth Analyzer is a universal device enabling to follow the lifecycle of algae or other biological objects producing oxygen. The device bears light source, exchangeable color filters, sensitive oxygen electrode and cover to model dark phase as shown in the Figure 33. It is controlled by Bioanalyzer potentiostat that allows user to program light and dark phases, measure and evaluate the oxygen electrode response. The device provides faster analogy of DIN 863 toxicity test that takes about 1 hour.

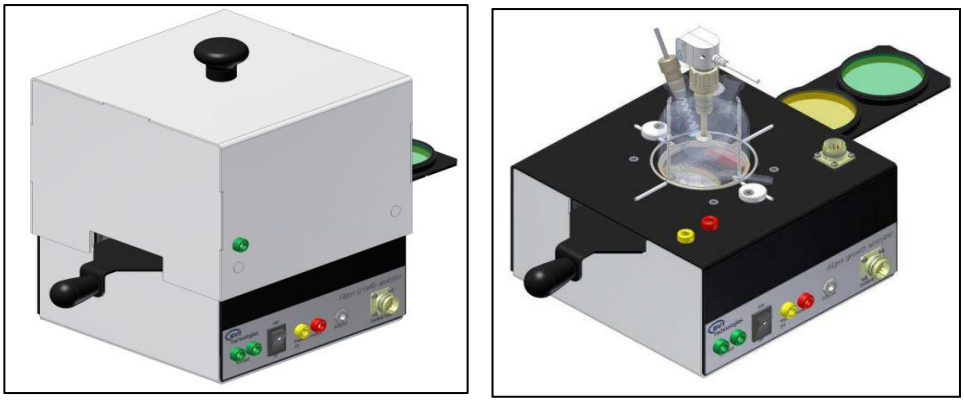


Figure 33: Algae Growth Analyzer equipment

3.3.3 Gas Chromatography coupled to Triple Quadrupole Mass Spectrometry

The use of chromatographic methods hyphenated with mass-spectrometric detection provides the excellent sensitivity and precision. GC-MS remains the most useful and sensitive method for the detection of trace levels of volatile pesticides. The most often used MS detector has been the quadrupole MS with EI. By utilising tandem MS it is possible to reach even lower detection limits. At present a combination of GC-MS and LC-MS/MS techniques appears to be the best approach to multi-compound class analysis [137, 138]. GC is among the earliest chromatographic techniques used for separation. GC has a number of advantages: (1) contaminants and impurities can usually be separated from the analytes; (2) the delicate choices of solvents, modifiers and gradient elution systems that characterise HPLC separations are not necessary for GC methods [139].

The Thermo Scientific TRACE 1310 Gas Chromatograph coupled with triple quadrupole mass spectrometry is used (Fig 34). The equipment features a complete icon-based touch screen interface which is ideal for direct instrument control when method development is required. The special features are auto sampler, increased robustness of injector technology and shorter sample cycle time.

Figure 34: TRACE 1310 Gas Chromatograph [140]

TSQ 8000 mass detector has the ability to analyze full scan data at the same time of targeted MRM analysis. In addition to simplified method start up, another advantage of using the analyzer is that it utilizes Timed-SRM methodology, which enables accurate pesticide identification and quantitation, even for very dense pesticide methodologies. The usability and scanning efficiency of Timed-SRM are complemented by the fast-scanning capability of the TSQ 8000 instrument, making the analysis of hundreds of pesticides, with a total of over one thousand transitions.

The instrument control and data processing software included with the TSQ 8000 mass detector, the use of the Trace Finder Pesticide Compound Database (CDB) greatly simplify the method development process.

By evaluating the retention times of target compounds, we are able to update the pesticides in the Pesticide Compound Database (CDB) with the known retention times. The software is capable of creating both the Trace Finder EFS processing method and the TSQ 8000 system Timed-SRM acquisition list, with acquisition windows centred on the retention times of the target peaks and allowing for acquisition window overlap, so that acquisition windows for all nearby eluting compounds are not forced to start and stop at the same time [140].

Chapter 4: Qualitative Analysis

For qualitative analysis two different techniques have been implemented. Both are discussed in detail as follows.

4.1 Method development utilizing Biosensors

The extensive use of pesticides to protect agricultural crops necessitates reliable tools for the detection of residues in food, water and other commodities, thus ensuring environmental protection and consumer safety. Neuro inhibitors such as organophosphates and carbamates in particular, represent a potential hazard to human health. Conventional methods of analysis are available but they are either time consuming or expensive.

Acetylcholinesterase (AChE) is a key enzyme in the nervous system. It terminates nerve impulses by catalysing the hydrolysis of neurotransmitter acetylcholine. As a specific molecular target of organophosphate and carbamate pesticides, acetylcholinesterase activity and its inhibition has been early recognized to be a human biological marker of pesticide poisoning. Measurement of AChE inhibition has been increasingly used in the last two decades as a biomarker of effect on nervous system following exposure to organophosphate and carbamate pesticides in occupational and environmental medicine.

A rapid, sensitive and low cost method based on AChE-inhibition utilizing biosensor was developed. The success of this biomarker arises from the fact that it meets a number of characteristics necessary for the successful application of a biological response as biomarker: the response is easy to measure, it shows a dose-dependent behaviour to pollutant exposure, it is sensitive, and it exhibits a link to health adverse effects.

4.1.1 Preparation of reagents and standards

MOPSO sodium salt was used for the preparation of buffer solution in BTA, where as Acetylthiocholine chloride (ATCh) as enzyme substrate and Neostigmine methyl sulfate as enzyme inhibitor.

The working solutions of individual pesticide standard Mix 155 (KF) was prepared by taking 10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) and analyzed in order of increasing concentration (Fig 35). Method blank (pure solvent) was also prepared as reference substance. The dilutions were made in different solvents i.e. hexane, dichloromethane and methanol. The working solutions thus constituted were stored in refrigerator.

Figure 35: Sample preparation

The prepared enzyme, substrate and standard inhibitor (Neostigmine methyl sulphate) must be stored at ± 4 ^{0}C during analysis so Termostat TK 1 (Fig 36, LHS) was used. The gentle stirring at different stages of analysis is necessary so a stirrer from Biocote® with different speed variation was also utilized (Fig 36, RHS).

Figure 36: Termostat TK 1 (LHS) and Stirrer (RHS)

4.1.2 Analytical parameters

One of the products of hydrolysis of ATCh with AChE is thiocholine (TCh). Detection of the change of the redox current of TCh (ΔI) can be used to assess the activity of AChE, which can be inhibited by OPs or Carbamates. In other words the inhibitory effect of different pesticides on AChE biosensors can be evaluated by determining the decrease in the current obtained for the oxidation of thiocholine. Thiocholine was

produced enzymatically by AChE using Acetylthiocholine (ATCh) as substrate (because thiocholine is not commercially available).

Thus the change of current of TCh after the biosensor was incubated with pesticides was linearly correlated with OPs concentration. The degree of inhibition was calculated as a relative decay of the biosensor response.

$$I\,\% = 100 \times \frac{I_O - I_i}{I_O}$$

Where I is the degree of inhibition of AChE; I_0 and I_i are the current values measured prior to and after the enzyme biosensor is treated with an inhibitor. There must be a certain positive correlation between *I* and the concentration of pesticides in principle [104, 141, 142].

4.1.3 Design of experiments

It is often necessary to investigate several different effects on a response of interest. A complete factorial experiment consists of an equal number of replicates for all possible combinations of the factors. There are many reasons for designing complete factorial experiment, and not for investigating one factor at a time: factorial experiments are much more efficient for estimating main averaged effects, secondly the interaction among factors can only be assessed in a factorial experiment (for instance, adsorption and extraction of each element separately). Interaction effects are important in the determination of the general conclusions over the experiment [143].

The classical approach of changing one variable at a time is today replaced with the optimizing of multivariable systems using experimental design. It has many advantages, like minimal number of experiments performed and the simultaneous determination of input level of responses. While applying the experimental design, numerous optimization experiments are not needed [144, 145].

All the above mentioned recommendations are taken into account while evaluating the performance of the developed method.

4.1.4 Electrochemical measurements

It has been fairly well established that the organophosphorus compounds, which are used agriculturally, may be efficiently detected using immobilized AChE. The

detection is based on the irreversible inhibition of the enzymatic activity in the presence of these organophosphorus and carbamates compounds.
AChE is an important enzyme which recognizes acetylcholine/acetylthiocholine and has been used to detect organophosphorus compounds based on the measurement of its percent inhibition in the presence of these pesticides [116].

The electrochemistry of acetylthiocholine and its enzymatic reaction product using AChE-immobilized biosensor has been described. The electrochemical measurements were performed at controlled room temperature (22 ± 1^0C) with BTA and Mini thermostat. The sensor AC1.W2.RS/AChE with platinum electrode was put into a Microflow system. The electrodes were connected to the Bioanalyzer. All measurements were performed at potential 350 mV. The interval between the additions was determined by stabilization of output current. The amperometric response was recorded throughout the process.

All the resultant extracts of classical cotton and organic cotton were tested on BTA. After putting the sensor in the slot the mopso buffer solution (8000 µl) was added in the Microflow unit. The background current was allowed to decay to a steady state followed by adding varying concentrations of Acetylthiocholine chloride. After stabilization the sample extracts were introduced in the Microflow unit and finally the inhibitor, Neostigmine methyl sulphate, was added into the solution to compare the inhibition of the sample with the standard inhibitor.

4.1.5 Preliminary Results

The activity of substrate (ATCh) with different concentrations (12.5, 25, 50,100 & 200) mM has been analyzed in stirred mopso buffer with the immobilized AChE bio sensors. It is evident from Figure 37 that with the increase of concentration the response is going higher. It indicates a relationship between these two variables.

Similarly the activity of standard inhibitor, Neostigmine methyl sulphate was analysed. After the addition of buffer and substrate, different amount of inhibitor was added with some interval (Fig 38) and we were able to see a more decline in the response of biosensor with the increase of the amount of inhibitor added. After final addition of inhibitor, the substrate was added again and it is quite visible that there is a considerable loss of activity if we compare it with the first addition of substrate. Even

by increasing the volume of substrate in the second addition we are not able to see a good response. It means that there is no sufficient enzyme left in the solution with which the substrate can react to form the product.

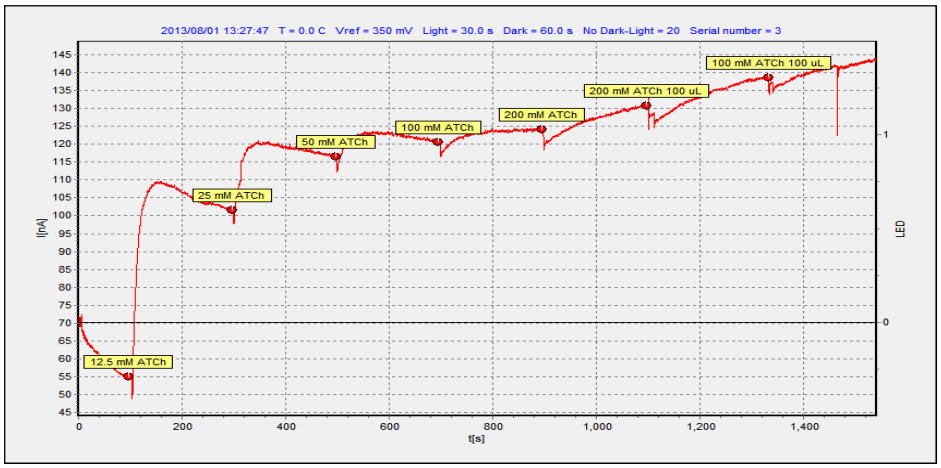


Figure 37: Response of sensor Vs substrate concentration

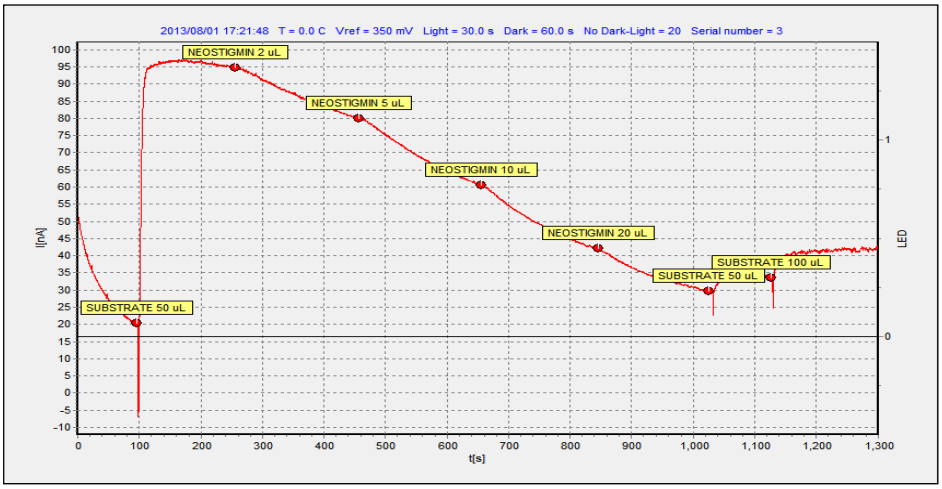


Figure 38: Response of sensor Vs STD Inhibitor concentration

In preliminary experiments, the final samples of cotton (PC, PO, GC, GO, IC, IO) which are extracted with hexane and dichloromethane were investigated. 8 mL of mopso buffer along with 25 mM ATCh was added in the Microflow unit and after some stabilization each individual sample was introduced. Finally the inhibitor is added.

It was observed that dichloromethane damaged the plastic part of the chamber of BTA where the sensor was placed so the use of this solvent was replaced by other organic solvents. Moreover we also compared the extraction time of ultra sound assisted method. The resultant graphs of the whole activity for PC and PO samples are shown in Fig 39 and Fig 40, respectively.

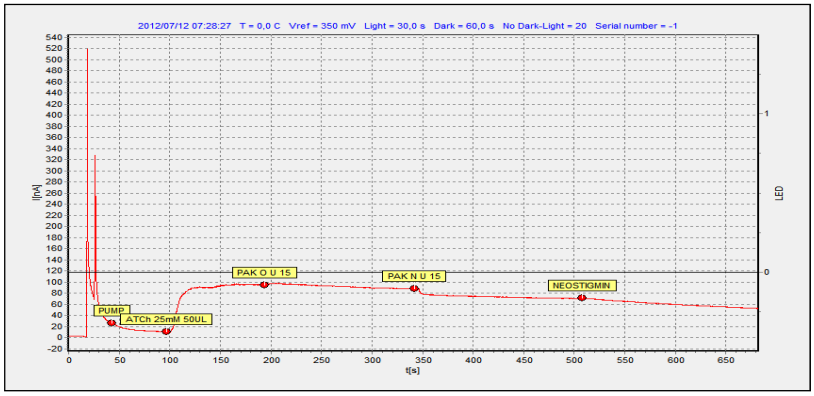


Figure 39: Amperometric response of PC samples (15 min)

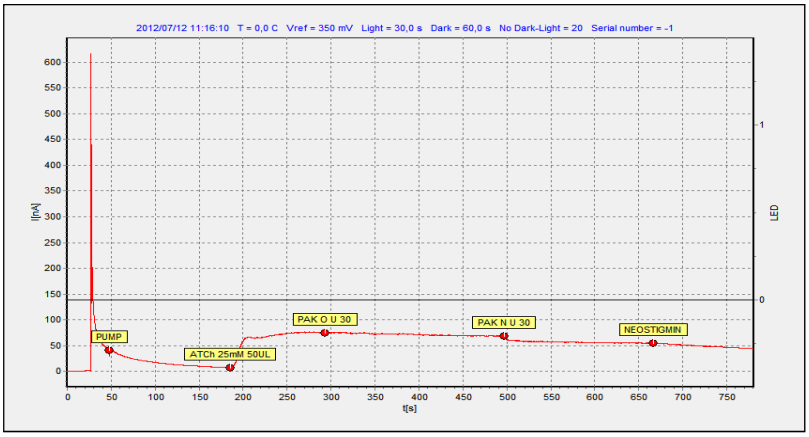


Figure 40: Amperometric response of PC samples (30 min)

Table 11 shows the detail of all the samples with two different timings and their relative inhibition. It is quite clear from this table that there is a substantial difference of inhibition between these timings. With 30 min extraction, we have better values so for the rest of the experiments; extraction with Ultra sound was being done with 30 minutes duration.

Table 11: Description of the preliminary results

Samples	**Extraction Time (min)**	**Classical Cotton**			**Organic Cotton**		
		Initial Current	**Current after Inhibition**	**Inhibition %**	**Initial Current**	**Current after Inhibition**	**Inhibition %**
Pak	15	88.22	76.66	13.10	95.74	88.22	7.85
	30	68.55	55.47	19.08	75.59	68.55	9.31
Giza	15	101.59	98.65	2.89	98.65	75.63	23.34
	30	83.68	70.04	16.30	76.54	61.96	19.05
Ind	15	97.60	90.00	7.79	90.00	70.22	21.98
	30	107.87	86.01	20.27	68.32	53.09	22.29

4.1.6 Calibration curves

10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) of Mix 155 (abbreviated as KF) were prepared and analyzed in order of increasing concentration. Method blank (pure solvent) was also prepared as reference substance. The dilutions were prepared in methanol. The same procedure as described earlier was implemented and instead of samples, we introduced the calibration points. We mentioned only the graphs of amperometric response of KFO (pure methanol) and KF 1000 in Figure 41 and 42, respectively.

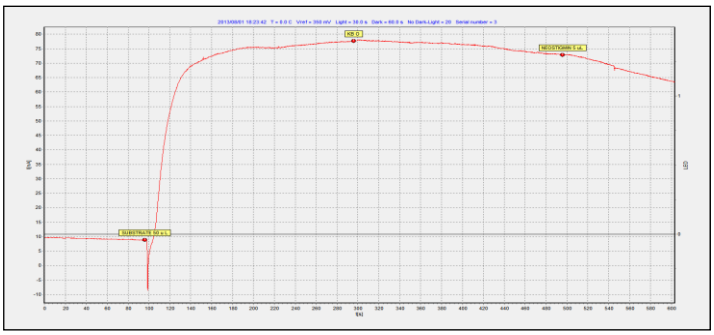


Figure 41: Amperometric response of KF 0 (pure methanol)

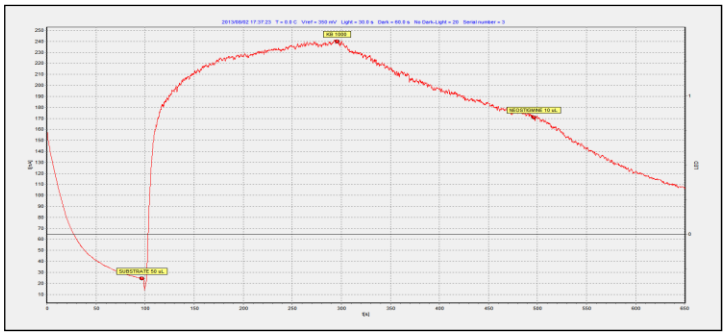


Figure 42: Amperometric response of KF 1000 ppb

As the calibration curve is a plot of detector response as a function of concentration [130], the activity of all the calibration points can be seen in Figure 43, where all the calibration points are plotted against the detector response (current).

On the basis of difference of current, Inhibition % was calculated and plotted against all the calibration points. With the increasing concentration from 0 ppb to 1000 ppb there is a trend of increment in inhibition % but with the variations in the middle section as shown in Figure 44. The initial slope of each concentration level is also plotted in the Figure 45. It is quite visible that there is a variation in the middle part of the curve. The data points follow a polynomial distribution.

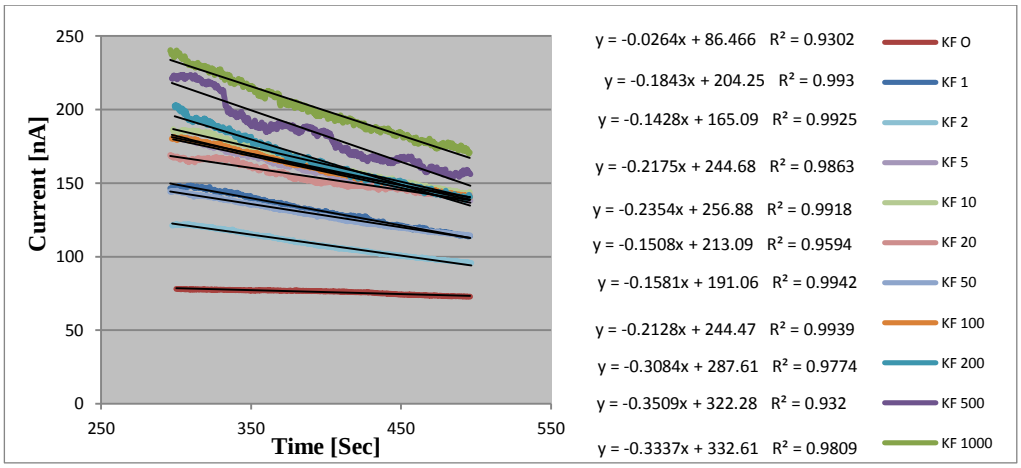


Figure 43: Amperometric response of all calibration points

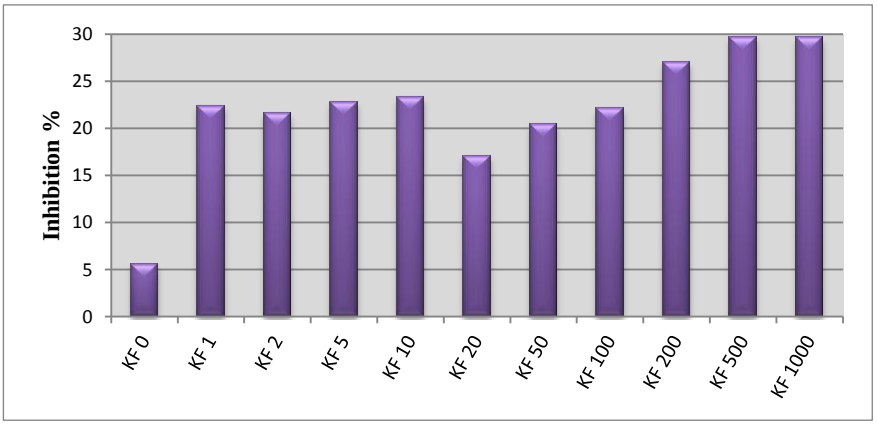


Figure 44: Inhibition % of all calibration points

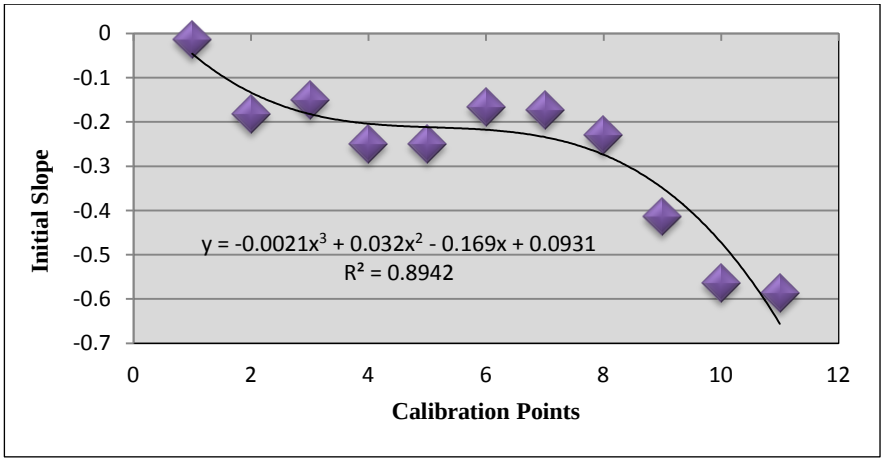


Figure 45: Initial slope Vs calibration points

To minimize these variations, the interaction of the calibration samples with the enzyme was exercised directly in the buffer and after 100 seconds, the effect of addition of the substrate was observed. AC1.W2.R1 sensor was used. The scheme of the experiment is shown below:

Time (Sec)	Addition	Volume (µL)
0	Buffer (Mopso Sodium)	8000
100	Enzyme (AChE)	2
200	Sample (Calibration Std)	50
300	Substrate (ATCh)	50

All the calibration samples have to unergo the same process and the resultant graphs were obtained. Figure 46 shows an example of KF 1000 ppb. All such graphs have been evaluated by measuring the initial slope of this curve, individually. The values of initial slope are plotted for all graphs against the relative calibration samples as shown in Figure 48.

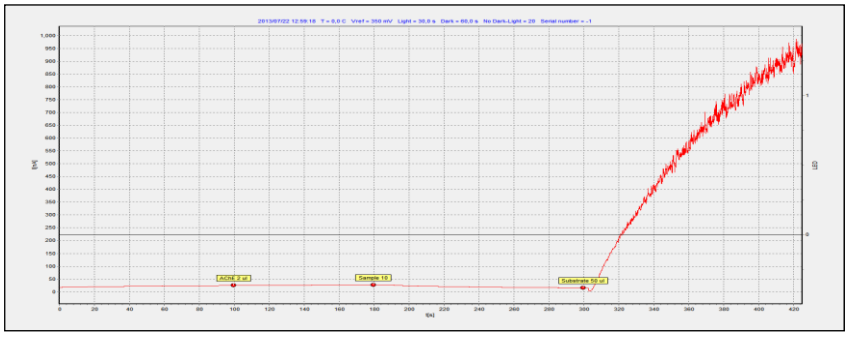


Figure 46: Amperometric response of KF 1000 ppb

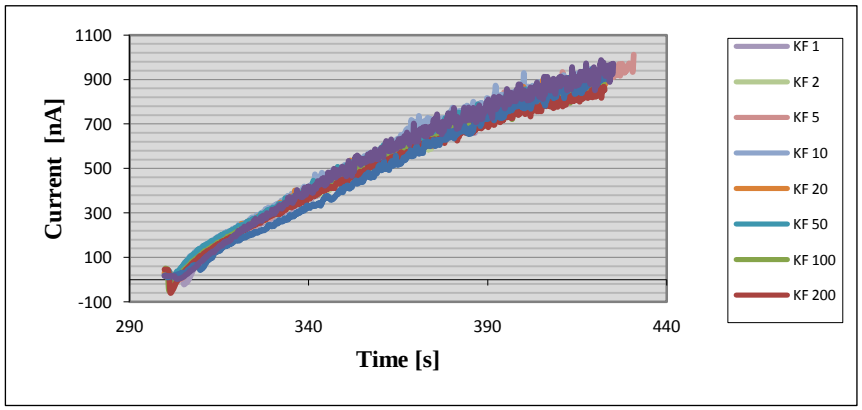


Figure 47: Amperometric response of all calibration points

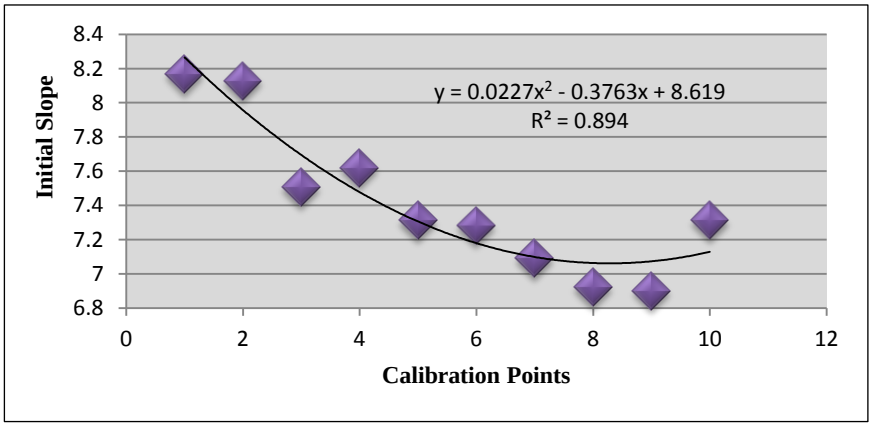


Figure 48: Initial slope Vs calibration points

We can see some better response in Figure 48. There is a smooth decline in the graph w.r.t. the increasing concentration but still the variation is there. One more intersting thing can be seen in Figure 47, where we are not able to see a significant difference of current between KF0 and KF1000.

Time (Sec)	Addition	Volume (µL)
0	Buffer (Mopso Sodium)	8000
100	Enzyme (AChE)	2
200	Sample (Calibration Std)	200
800	Substrate (ATCh)	50

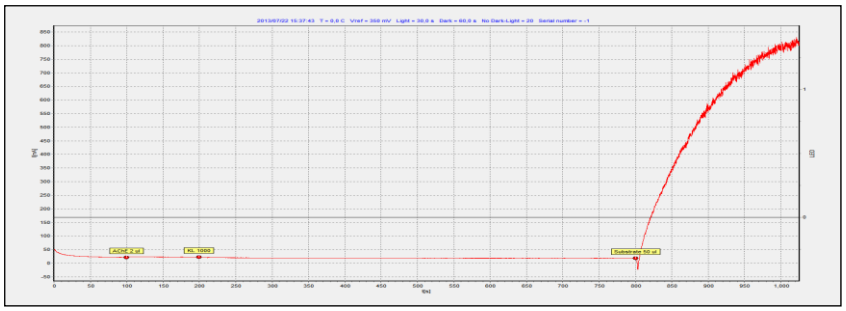


Figure 49: Amperometric response of KF 1000 ppb

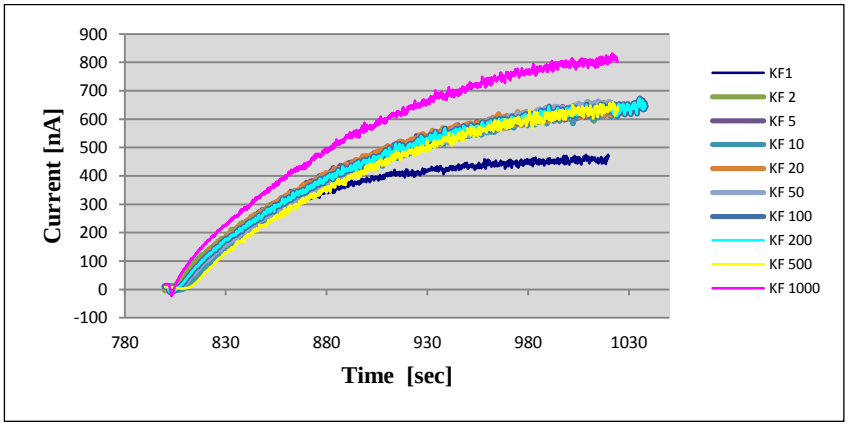


Figure 50: Amperometric response of all calibration samples

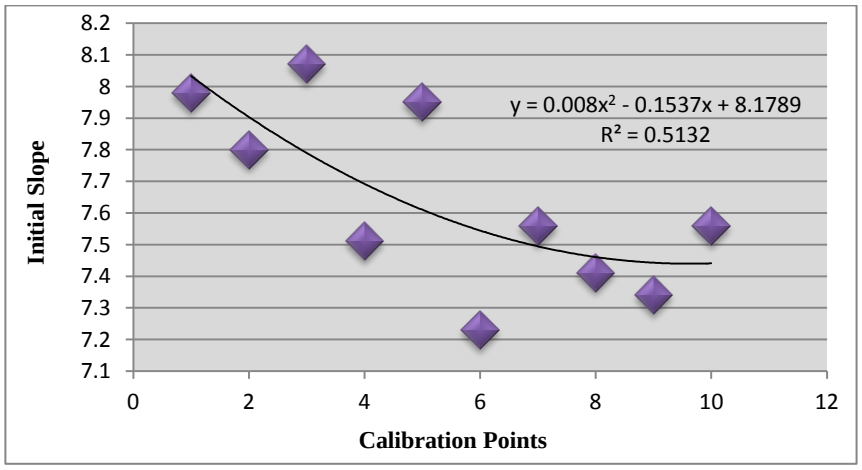


Figure 51: Initial slope Vs calibration points

By increasing the time of incubation with the calibration points, we can see a significant difference of current (Fig 50) between the Lowest Calibration Level (LCL) and Highest Calibration Level (HCL) but there is a huge variations in the graph of initial slope of the curves plotted against the calibration levels (Fig 51).

In a seperate vial, enzyme was incubated with the sample for 300 seconds and then put in the buffer solution. The substrate is added after 100 seconds and the activity was monitored. We are not able to see a smooth trend, the variation is still obvious (Fig 52).

Time (Sec)	Addition	Volume (µL)
0	Buffer (Mopso Sodium)	8000
100	[Enzyme (AChE) (2 µL) + 20 µL of calibration sample]	
400	Sample (Calibration Std) + Enzyme	15
500	Substrate (ATCh)	50

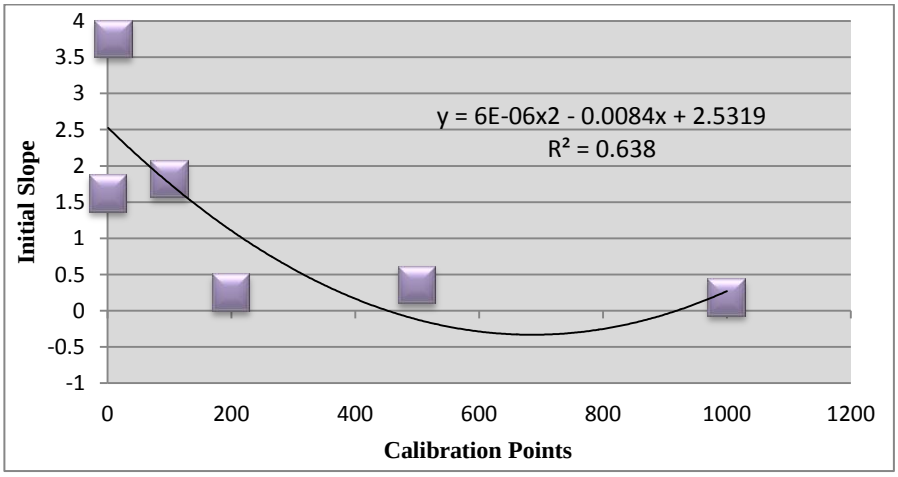


Figure 52: Initial slope Vs calibration points

We tried to interact our LCL (KF 0) and HCL (KF 1000) with distilled water alongwith enzyme for 300 seconds and then put it in the buffer solution and finally the substrate was added.

Time (Sec)	Addition	Volume (µL)
0	Buffer (Mopso Sodium)	8000
100	Enzyme (AChE) (2 µL) + 20 µL of (calibration sample + DW 50 µL)	
400	Aq.Sample (Calibration Std)	15
500	Substrate (ATCh)	50

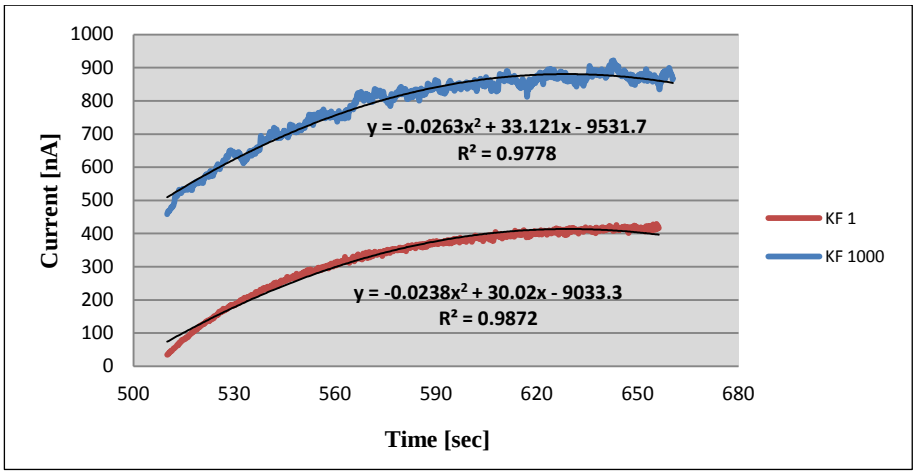


Figure 53: Amperometric response of KF 1000 ppb

Figure 53 shows the amperometric response of KF 0 and KF 1000. We can see a significant difference of current and a good relation but the initial slope difference is not so huge i.e. the initial slope for KF 1 is 8.708 and the slope for KF 1000 is 9.038.

The above mentioned basic methods have been implemented to have an idea of the enzyme kinetics. We have observed that the interaction of the analyte (calibration STDs) with the enzyme is not proper; otherwise we definitely must have a good correlation between them. First reason may be the difference of phases, as our analytes (calibration STDs) were prepared in an organic solvent where as the enzyme and buffers are in aqueous solution. Second reason may be the inappropriate concentration of enzyme and the substrate. In the consequent experiments the above mentioned factors will be considered.

4.1.7 Enzyme activity

The enzyme activity has been analyzed following the method adopted by George L. Ellman, in which the determination of acetylcholinesterase activity was measured by following the increase of yellow colour produced from thiocholine by a photometric method [146].

We adopted only the methodology of the above mentioned method. The testing was performed on Minithermostat MT-1 which is a modified version of Biosensor Toxicity Analyzer (BTA). Different Enzyme Concentration like (1, 0.1, 0.01 IU) and substrate concentration (100, 50, 25 mM) have been analyzed. The scheme of the experiment was as under:

	Substrate Concentration		
Enzyme Concentration	**100 mM**	**50 mM**	**25 mM**
1 IU	√		
0.1 IU	√	√	
0.01 IU	√		√

100 µL of mopso buffer, 100 µL of ATCh (with varying concentrations as described earlier) and 100 µL of Calibration standards (individually) were added in the separate vials. After gentle stirring, Acetylcholinesterase (AChE) was added and exactly after ten minutes 10 µL Neostigmine was added. The vials are stirred and centrifuged for 1 minute each.

The time of incubation with enzyme is a critical step so special attention was paid so that each sample has an equal chance of interaction with the enzyme. After the addition of inhibitor, all the calibration samples were analyzed. One of the resultant graphs of the whole activity is shown in Figure 54 with enzyme concentration of 0.1 IU and substrate of 50 mM. The difference of current for each calibration point was evaluated from these graphs and plotted as can be seen in the Figure 55 with a combination of different enzyme and substrate concentration. The variation can be seen in these graphs. With a deep study of all these graphs, it has been established that at lower concentration of substrate, we have a good correlation between these two variables.

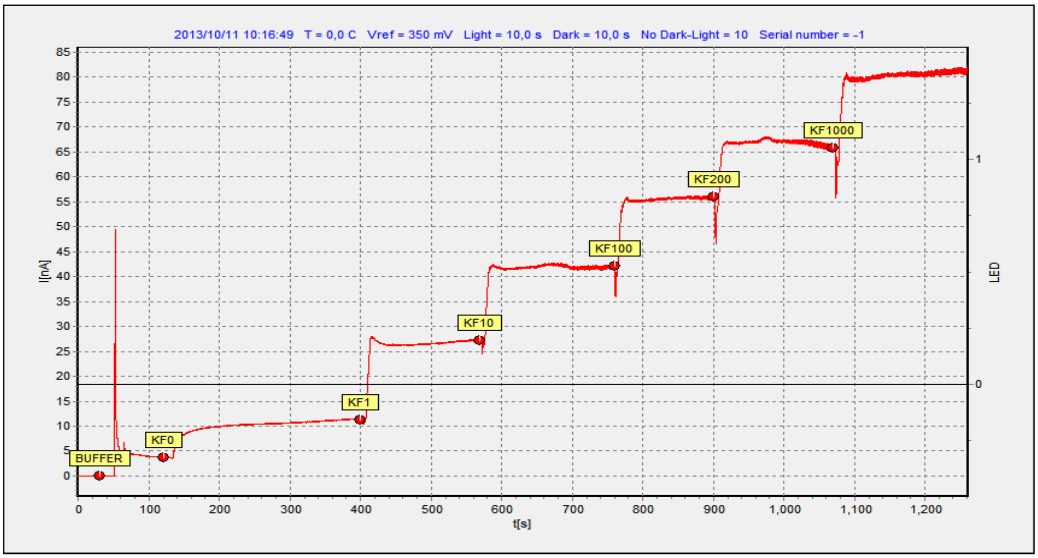


Figure 54: Detector response with 0.1 IU enzyme and 50 mM of substrate

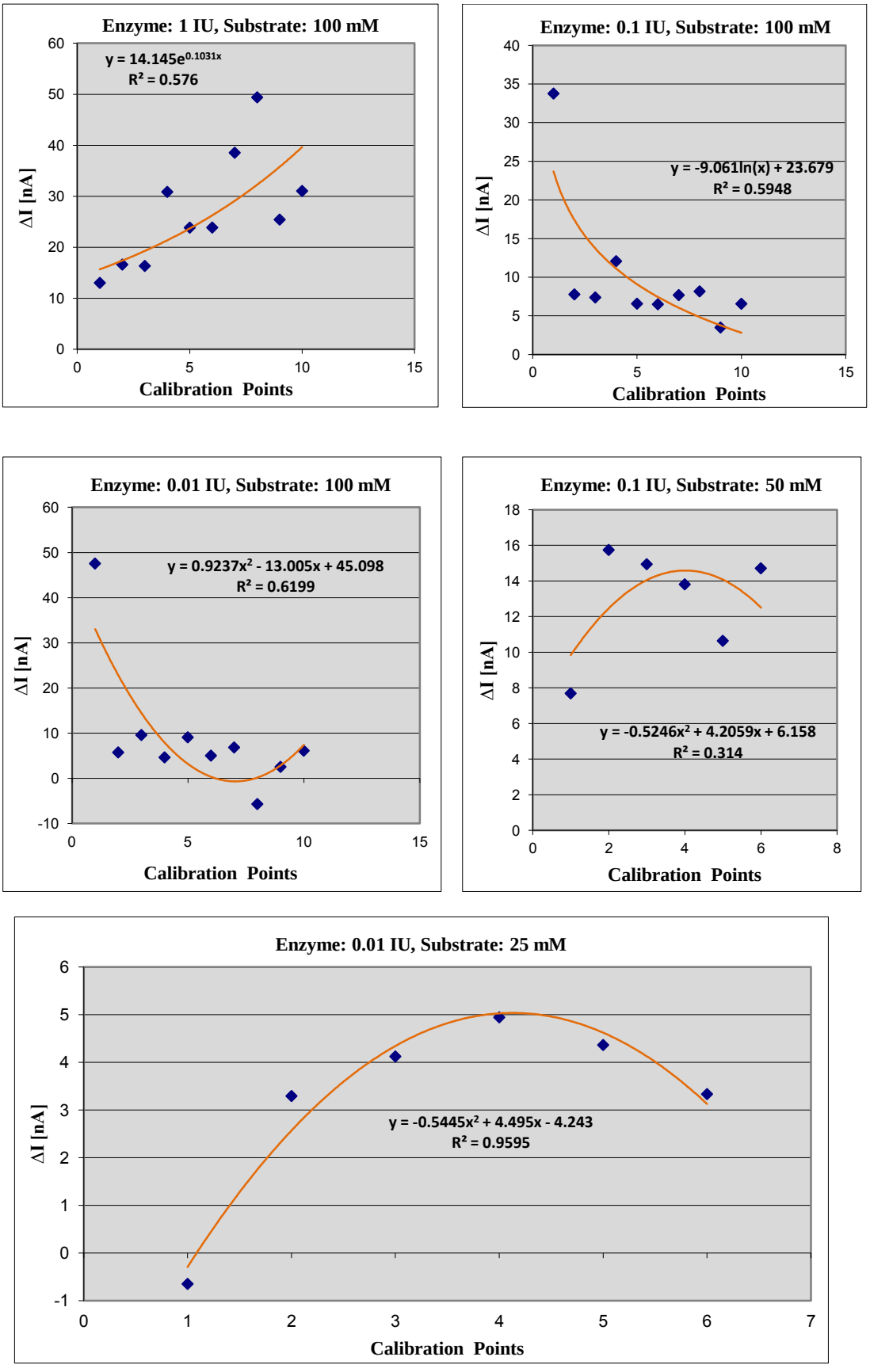


Figure 55: AChE Enzyme activity with different concentrations

4.1.8 Impact of Phase Catalyst Agents

Two different phase transfer catalysts i.e. Tetrabutyl ammonium hydrogen sulfate and Tween were used. TECHNIC 1 Linear Pump was used which is responsible for a continuous flow of buffer and also carry the injected analytes on to the surface of biosensor.

Different concentration (62.5, 15, 6.25 and 0.625 mM) of Tetrabutyl ammonium hydrogen sulphate (TBAHS) is added in 100 µL of mopso buffer solution (MBS). 100 µL of ATCh (50 mM) and 100 µL of Calibration standards (KF0, KF1, KF10, KF 100, KF1000) were added in the separate vials. After gentle stirring, Acetylcholinesterase (AChE) was added and exactly after ten minutes 10 µL Neostigmine was added. The vials are stirred and centrifuged for 1 minute each. The results are shown in Figure 56.

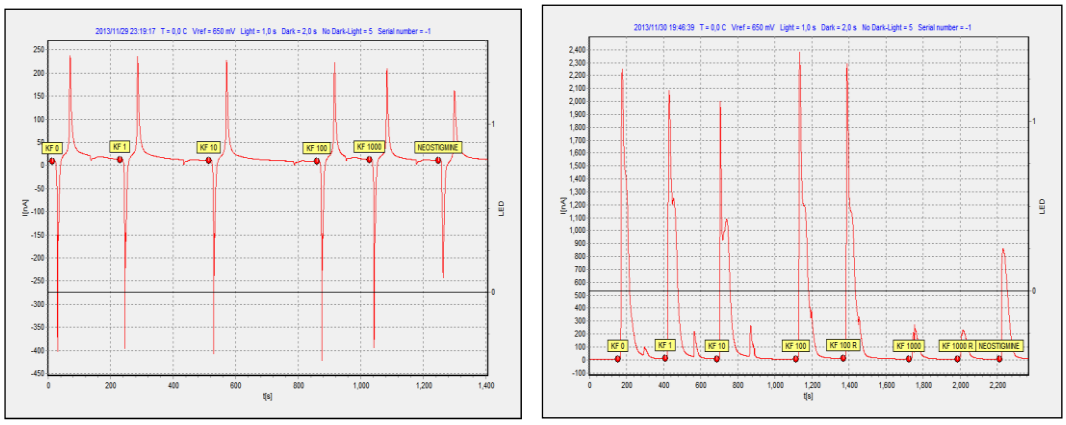


Figure 56: Amperometric response with TBAHS 62.5 mM & 0.625 mM

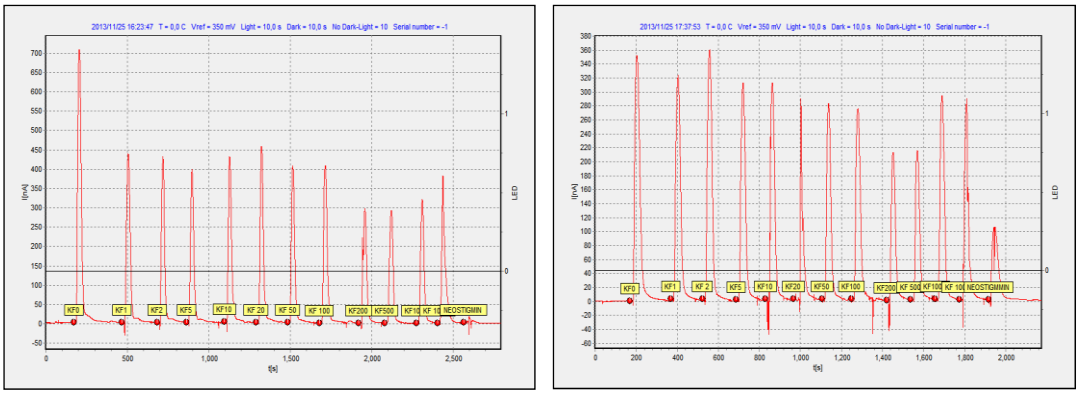


Figure 57: Amperometric response with Tween 1% (LHS) and 0.1 % (RHS)

Above mentioned procedure is repeated for tween with concentrations of 1% and 0.1 % in the MBS. The resultant graphs are shown in Figure 57. It has been observed that the use of phase catalyst agents is not helpful in this case. The poor correlation between the calibration samples and the detector response was observed. Another effort was being done by evaporating the solvent methanol of calibration samples in vials and same

procedure was followed as mentioned earlier. The result of this activity is shown in Figure 58. The response is not satisfactory even with the elimination of the organic solvent.

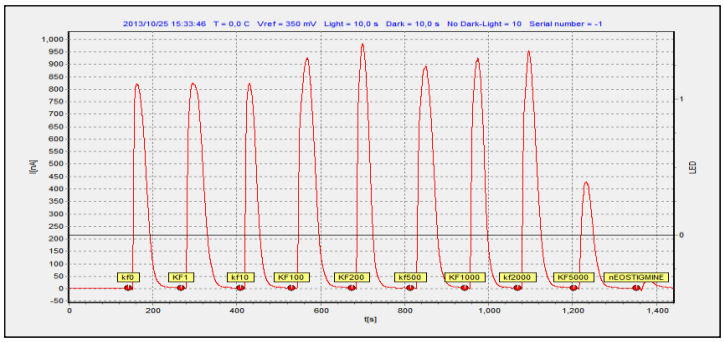


Figure 58: Amperometric response by evaporating the solvent

4.1.9 Effect of pH and Buffers

Mopso buffer solution (MBS) was prepared with 6.0, 7.0 & 8.0 pH values. 100 µL OF MBS with different pH values was put in the vials separately. 100 µL of ATCh (50 mM) and 100 µL of Calibration standards (KF0, KF1, KF2, KF5, KF10, KF20, KF50, KF100, KF200, KF 500, KF1000, KF2000 & KF5000) were added step wise.

After gentle stirring, Acetylcholinesterase (AChE) was added and exactly after ten minutes 10 µL Neostigmine was added. The vials are stirred and centrifuged for 1 minute each. The analytes were tested with biosensor and the amperometric responses at these pH values are shown in Figure 59 and Figure 60.

There is variation of the response at all pH values. At pH 7 higher concentration of the calibration points show a smooth decline. Although this decline is still not in a regular manner but we can observe the huge difference of KF200 and KF1000 values.

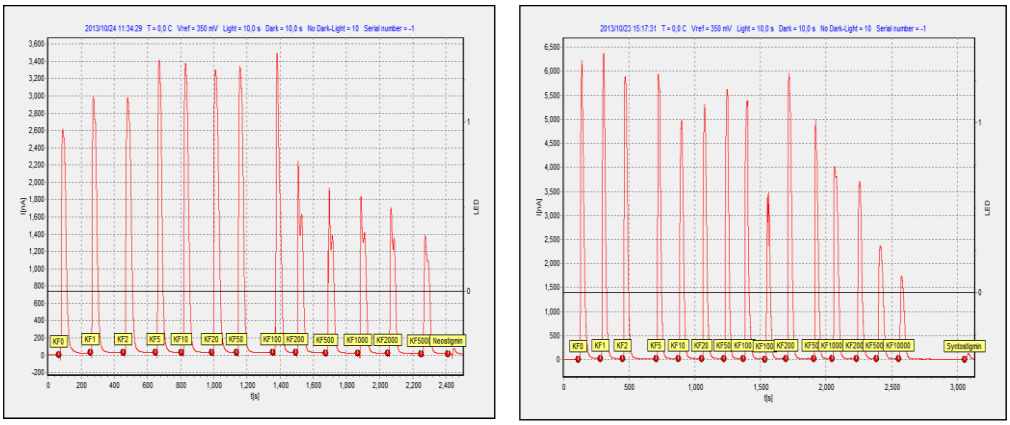


Figure 59: Detector response for pH 6 (LHS) & pH 7 (RHS)

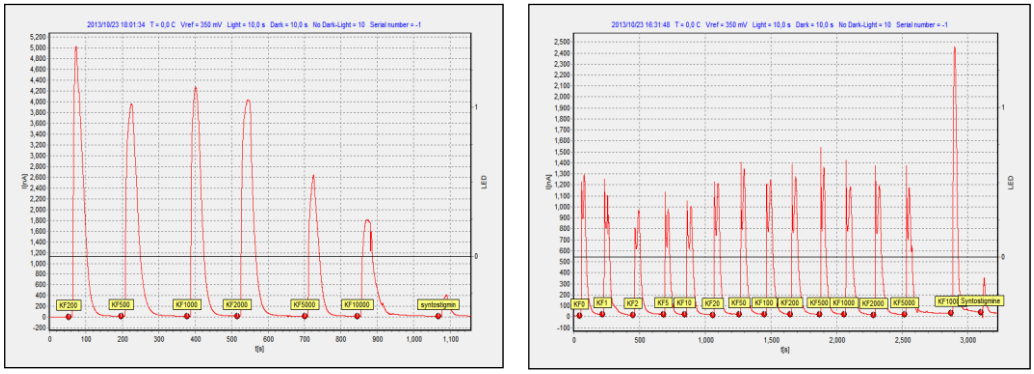


Figure 60: Detector response for pH 7 (Higher Calibration points) (LHS) & pH 8 (RHS)

As Phosphate buffer was claimed to be the most suitable buffer among four different buffers for the investigation of interactions between AChE and OP compounds in a study by T. Wille [147].

Mopso Buffer solution (MBS) was compared with Phosphate Buffer solution (PBS) with the same methodology as being applied for different pH values. As we have better results of Mopso Buffer with pH 7 so we tested both buffers with pH value 7 for Mopso and 7.3 for Phosphate buffer. The results are shown in Figure 61. It is obvious that phosphate buffer's response is quite logical. The response is declining with the increase of analyte concentrations.

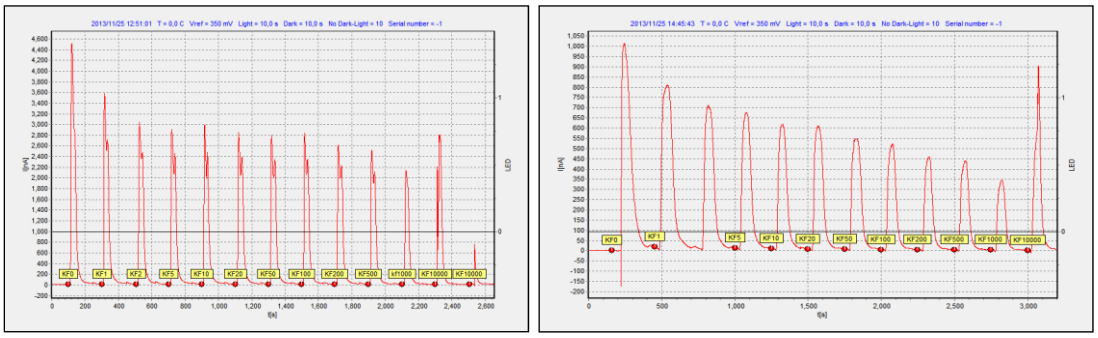


Figure 61: Amperometric response for Mopso Buffer (LHS) & Phosphate Buffer (RHS)

Phosphate buffer was preferred for the next experiments as some fine tuning is still needed for optimization.

4.1.10 Optimization of Enzyme and Substrate concentration

As it was established from our earlier results that lower concentration of substrate is more responsive so we have prepared different lesser concentrations (0.08, 0.16, 0.32, 0.64, 0.8 mM) of ATCh with the addition of 1M of $MgCl_2$ and 2.5M of NaCl in phosphate buffer. The enzyme Acetylcholinesterase from electric eel (EC 3.1.1.7) was

prepared by dissolving 0.6 mg of AChE in 0.4% BSA (Bovine serum albumin) containing 0.1M phosphate buffer with pH value 7.3. The final concentration of AChE prepared was 0.5 IU/µL.

The use of BSA into the solution of AchE enzyme was found to generate the most sensitive responses and also helpful in decreasing the detection limit for quantification of pesticidea analysis as reported by [148, 149]. The same procedure was implemented as described before and the response of the calibration points was observed. The final results are shown in Figure 62. It is quite visible from this graph that we have got the maximum response with the minimum concentration of the substrate i.e. 0.08mM.

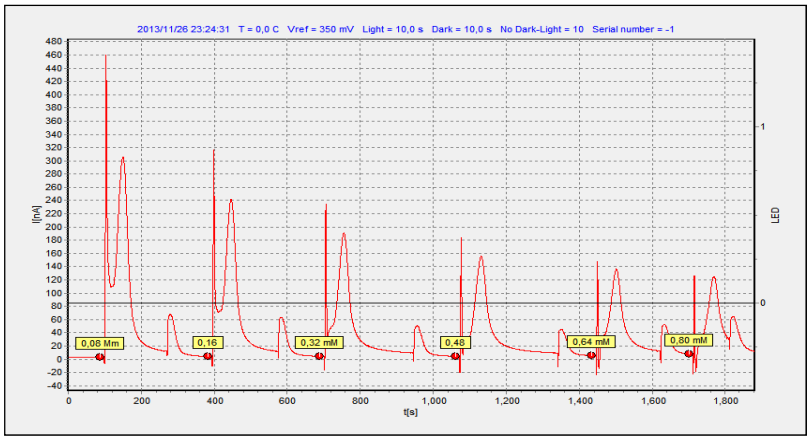


Figure 62: Optimization of substrate concentration

4.1.11 Effect of Incubation Time

The time of incubation has a significant role in AChE inhibition mechanism. As all the previous tests were analyzed with 10 minutes incubation with AChE so to optimize this variable, we have made four different test with different timing of incubation (10, 30, 60 & 180 min) of enzyme and calibration STDs. The resultant graphs are shown in Figure 63 & Figure 64.

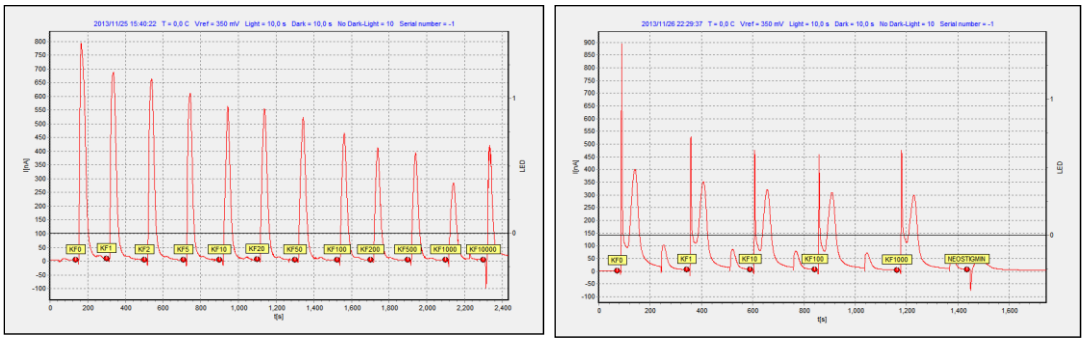


Figure 63: Incubation with AChE for 10 min (LHS) & 30 min (RHS)

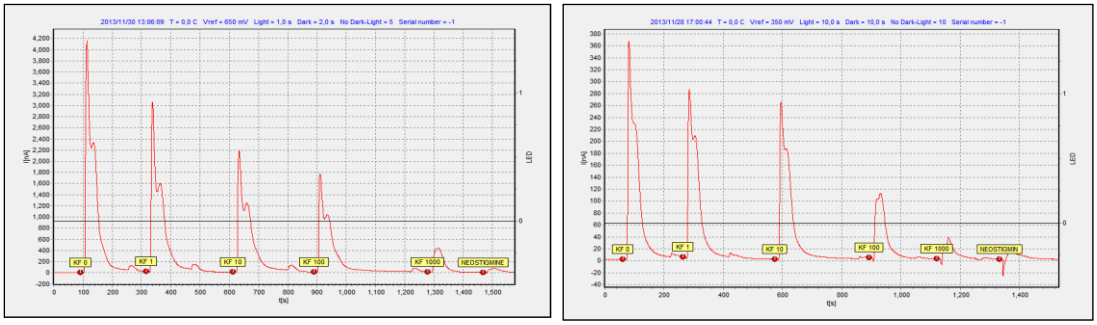


Figure 64: Incubation with AChE for 60 min (LHS) & 180 min (RHS)

It has been observed that the incubation for 60 minutes of calibration points with AChE is the most appropriate. We have got a good correlation between these two variables which is logical. The highest response is with pure methanol (KFO) which means that AChE is least affected by this analyte, where as in case of standard inhibitor (Neostigmine) the response is very low representing a major inhibition of AChE enzyme.

4.1.12 AChE Inhibition Activity

All the variables involved in AChE inhibition activity have been studied and optimized. The prepared calibration standards (KF0, KF1, KF10, KF100 & KF1000) were analyzed for AChE inhibition. The scheme of the final testing is described in Table 12.

Table 12: Scheme of testing

Addition of Substances	**Volume (µL)**
0.1M Phosphate Buffer	100
ATCh (0.08 mM)	100
Sample (Calibration Std)	100
Stirring	
AChE (0.5 IU/ µL)	2
Stirring	
After 60 minutes	
Neostigmine	10
Final Stirring	

The results of the above mentioned procedure are shown in the Figure 65. A good correlation between AChE activity and the calibration points was observed. The relevant peak current against calibration points was plotted as shown in Figure 66. The data points follow a quadratic trend which is more or less like the behaviour of dose-response curve.

The area under these consecutive curves have been evaluated and plotted in the graph (Fig 67). A smooth decline was quite clearly observed.

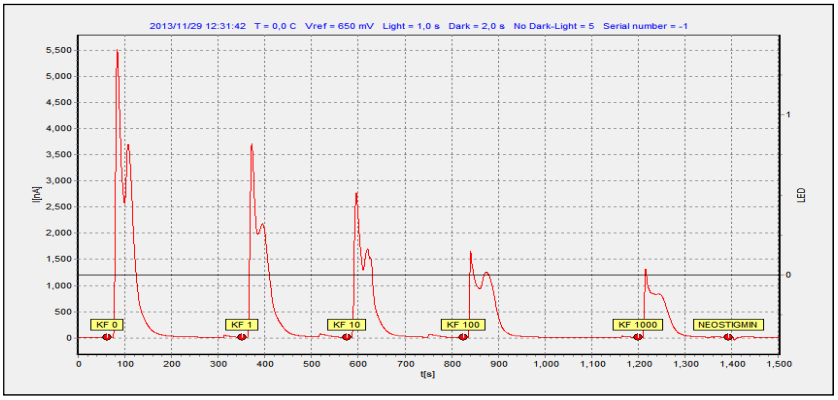


Figure 65: Amperometric response of calibration samples with optimized concentrations

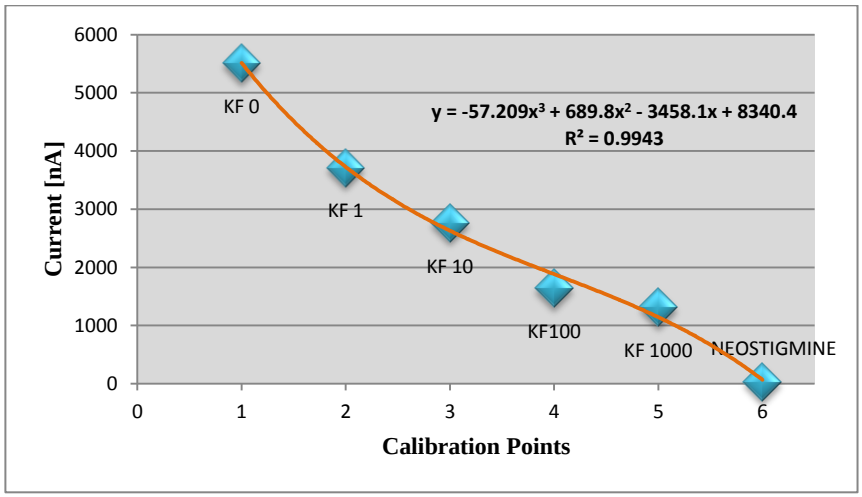


Figure 66: Current Vs Calibration Samples

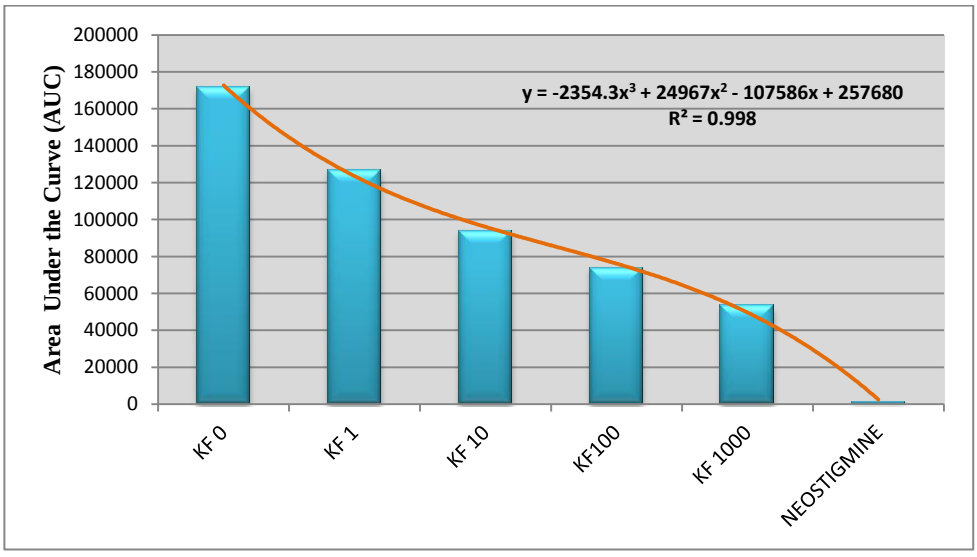


Figure 67: Area under the curve for Calibration Samples

Five repititions (A, B, C, D, E) utilizing the above mentioned method have been exercised and the resultant graphs are shown in the Figure 68, where as the area under the curves of all these graphs is summarized in Figure 69.

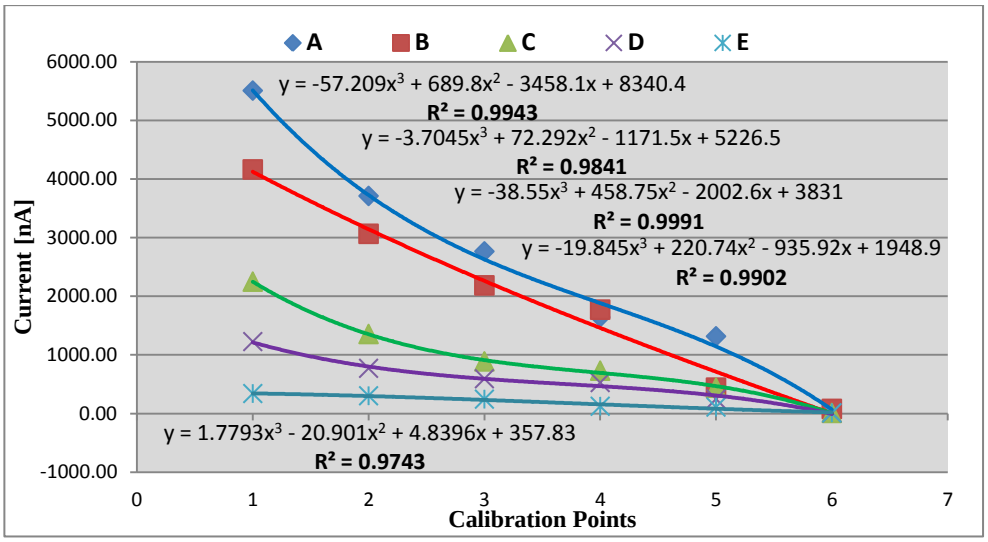


Figure 68: Amperometric response of different calibration samples; *n=5*

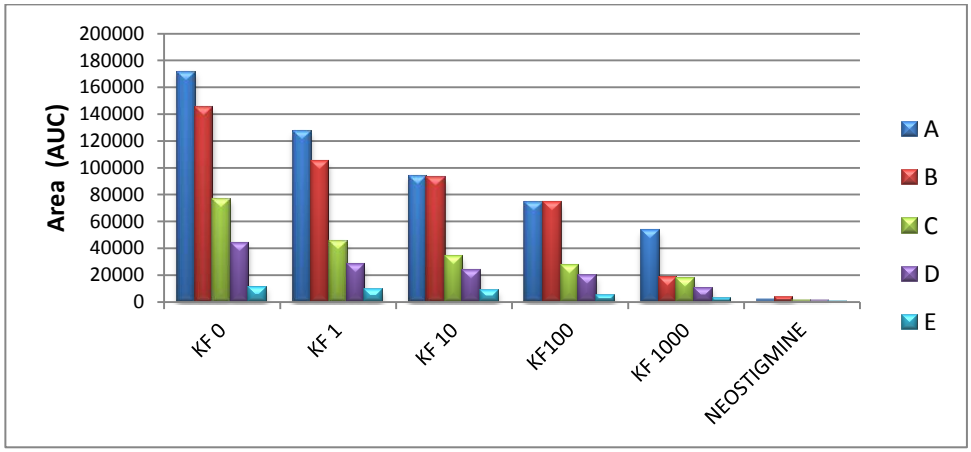


Figure 69: AUC for different calibration samples; *n*=5

Another approach for determining the AChE inhibition % was investigated by Arvinte & Skladal [13 & 150]. The standard curve method was used for quantitative detection utilizing differential pulse voltammetry (DPV). The inhibition percentage was calculated using the equation:

$$I\,\% = 100 \times \frac{A_o - A_i}{A_o}$$

Where I (%) represent the inhibition percentage, A is the DPV peak area. The subscripts (0) and (1) correspond to measurements performed before and after incubation, respectively.

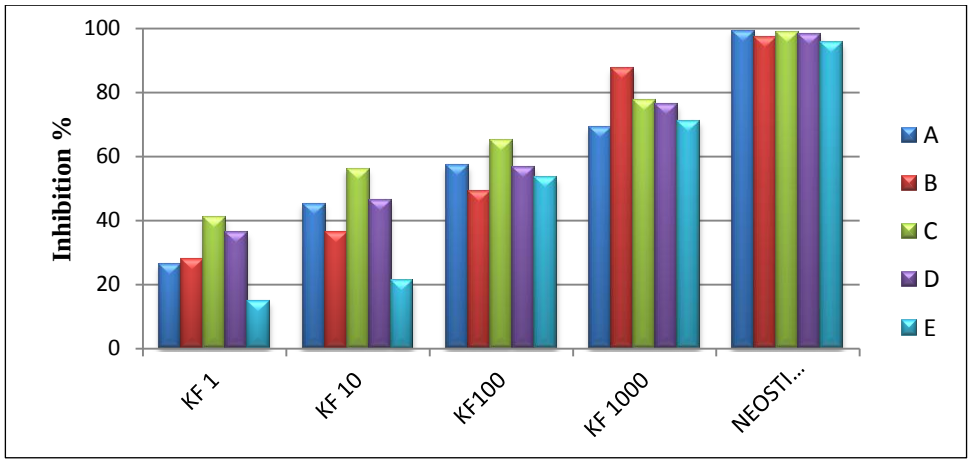


Figure 70: AChE-inhibition caused by different concentrations; *n*=5

The inhibitions % was calculated utilizing the above mentioned equation for all the repititions and the detail is shown in Table 13. All the important parameters like Standard Deviation, Standard Error, Mean, R.S.D, Confidence Level (95.0%) have been calculated.

Table 13: Summary of Inibition % and other parameters for all the repititions

		Calibration Points				
		KF 1	**KF 10**	**KF100**	**KF 1000**	**NEOSTIGMINE**
Inhibition %	A	*26.13*	*45.20*	*57.06*	*69.07*	*99.24*
	B	*27.78*	*36.22*	*49.19*	*87.50*	*97.27*
	C	*40.93*	*56.03*	*64.89*	*77.43*	*98.89*
	D	*36.13*	*46.35*	*56.58*	*76.21*	*98.13*
	E	*14.83*	*21.11*	*53.45*	*71.06*	*95.55*
Mean		29.16	40.98	56.23	76.25	97.82
Standard Error		4.49	5.88	2.58	3.21	0.66
Median		27.78	45.20	56.58	76.21	98.13
Standard Deviation		10.04	13.14	5.77	7.18	1.48
Sample Variance		100.89	172.66	33.27	51.61	2.18
R.S.D		34.45	32.06	10.26	9.42	1.51
Kurtosis		-0.16	0.76	1.19	1.10	0.41
Skewness		-0.41	-0.79	0.60	1.03	-0.99
Range		26.10	34.92	15.70	18.43	3.69
Minimum		14.83	21.11	49.19	69.07	95.55
Maximum		40.93	56.03	64.89	87.50	99.24
Sum		145.80	204.91	281.17	381.27	489.08
Count		5.00	5.00	5.00	5.00	5.00
Confidence Level (95.0%)		12.47	16.32	7.16	8.92	1.83
Upper Bound		41.63	57.30	63.40	85.17	99.65
Lower Bound		16.69	24.67	49.07	67.33	95.98

It is obvious from the Table 13 and Figure 70 that at lower concentrations, higher values of RSD have been observed but as the concentration increses this parameter appers to be going less and less so as the standard error and confidence level. The results also show that the method developed by this study shows a good tendency for repeatability. The method can be used for measuring the inhibition % of the extracts from cotton samples.

The detection limit, defined as the concentration of pesticide that produce an inhibition percentage of 10% of the AChE activity as reported by [13, 151, 152, 153, 154]. Figure 71 shows a graph between the AChE average inhibition percent caused by the different concentrations of calibration samples. From this graph we are able to see that in this case, the detection limit is in the range of below 1 ppb.

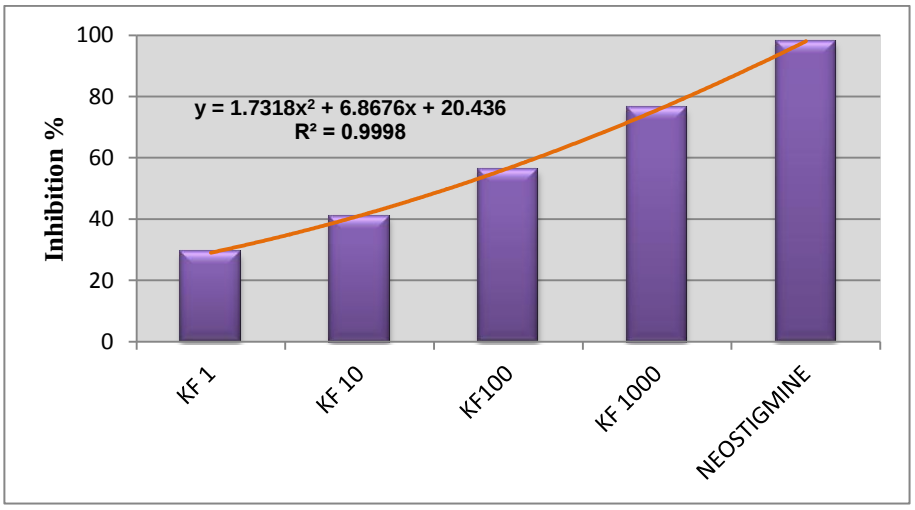


Figure 71: Average AChE-inhibition caused by different concentrations; *n*=5

The equation of the best fit line is as follows:

$$y = 1.7318x^2 + 6.8676x + 20.436$$

The value of predicted squared coefficient of correlation (R^2) is found to be 0.9998, which is excellent and shows a strong relationship between our variables i.e. Concentration and Inhibition %. The other regression statistics and ANOVA characteristics have been calculated for all these repetitions and are shown in the Table 14.

Table 14: Description of regression analysis

Regression Statistics	
Multiple R	0.9999
R Square	0.9998
Adjusted R Square	0.9996
Standard Error	0.5747
Observations	5

	df	**SS**	**MS**	**F**	**Significance F**	
Regression	2	3020.29	1510.14	4572.31	0.00022	
Residual	2	0.66	0.33			
Total	4	3020.95				
	Coefficients	**Standard Error**	**t Stat**	**P-value**	**Lower 95%**	**Upper 95%**
Intercept	20.426	1.233	16.572	0.0036	15.123	25.729
Cal Points	1.73	0.154	11.263	0.0078	1.069	2.391
Cal Points	6.878	0.939	7.322	0.0181	2.836	10.920

The residuals have been plotted against the different concentration levels and shown in Figure 72, whereas Figure 73 shows a comparison of measured and predicted inhibition % based on the regression model. It is quite obvious from Figure 72 that the residuals are scattered randomly and there is not a pattern or trend which can be seen in the data set of residuals.

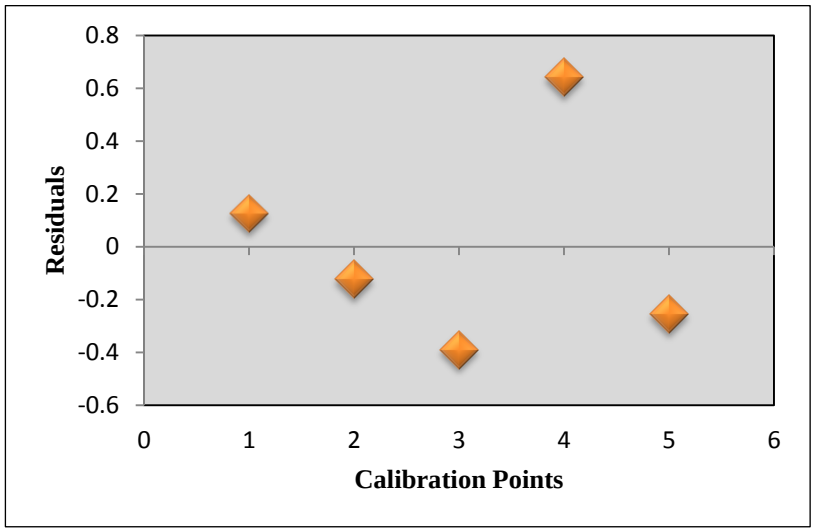


Figure 72: Residuals plot

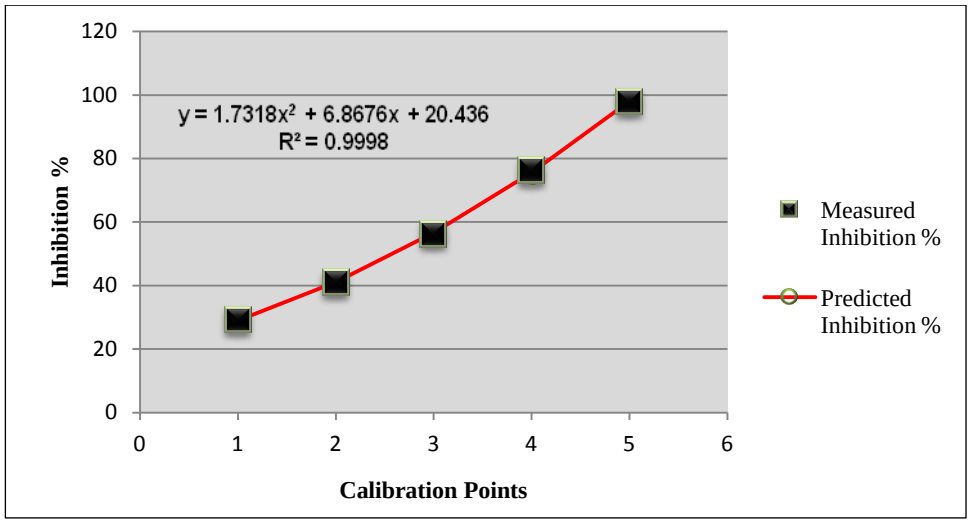


Figure 73: Measured and Predicted Inhibition % from regression model

It is worth noting that not only different compounds may reach levels of significance in terms of anticholinesterase effect, but, moreover, combinations of different chemical classes can exert additive or synergistic inhibitory effect on AChE activity [155]. As Mix 155 contains about 18 different pesticides so the inhibition percent observed by each concentration may be attributed of this synergistic effect. This also suggests that for quantification individual pesticide standards must be utilized.

4.1.13 Method Application

The method is utilized for real cotton samples extracted with different solvents (methanol, hexane, toluene, acetone & acetonitrile) after necessary sample pretreatments. The speciality of this method is that all the samples along with the control points can be tested in one run, The total time utilized for one complete test was approximately 50 ~ 55 minutes.

Firsly, the activity of all the solvents was observed as shown in Figure 74. Other than acetone and acetonitrile, all solvents show a reasonable response. All the samples were prepared by the procedure mentioned in Table 12. Instead of calibration samples, the extracts of cotton samples were inducted. After the complete procedure these final samples were introduced to the biosensor and the response is monitored. Figure 75 shows the activity of whole the experiment and Figure 76 shows the graph which was plotted against the AUC and corresponding analytes.

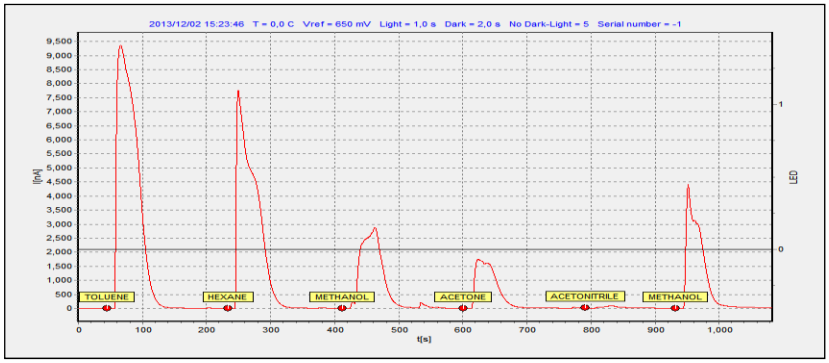


Figure 74: Amperometric response of different solvents

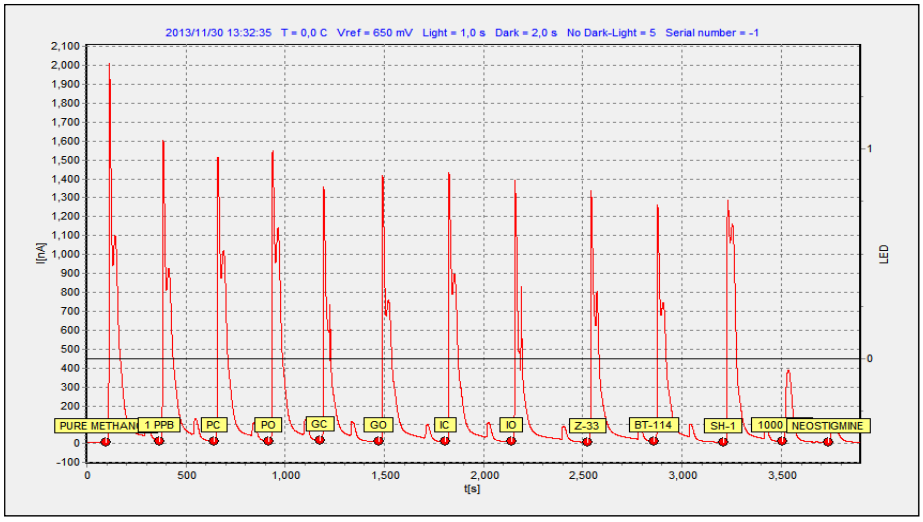


Figure 75: Amperometric response of all cotton samples extracted with methanol

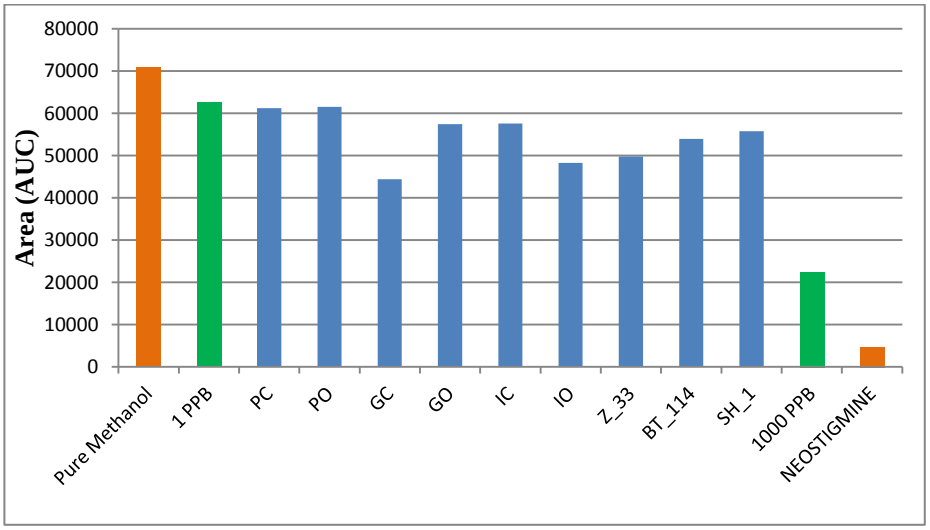


Figure 76: Area (AUC) of all cotton samples

We are able to compare our extracts with the help of minimum and maximum concentration‘s area. It is quite visible that almost all our samples have the area with in the range of 1 ppb to 1000 ppb but none of the samples exceed 1000 ppb limit.

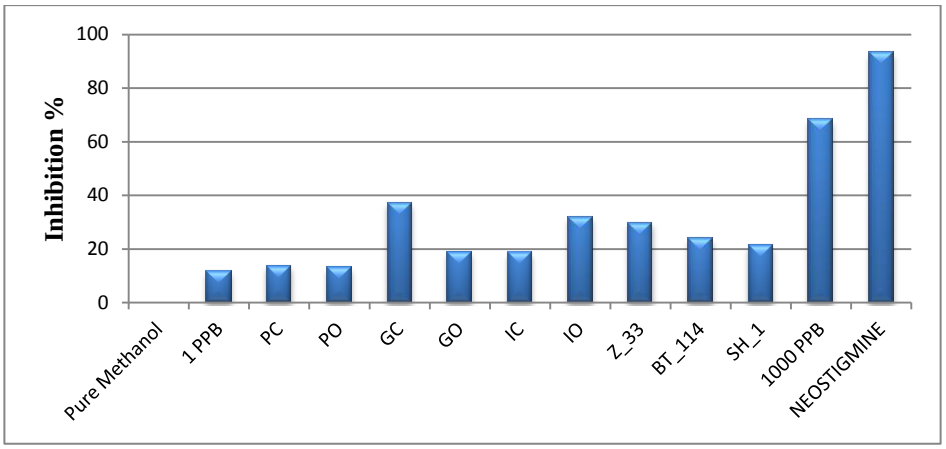


Figure 77: AChE-Inhibition caused by all cotton samples extracted with methanol

The inhibition % was calculated based on the area under the corresponding curves for each analyte and represented in Figure 77. It shows that all of our samples show the inhibition % (on average of < 40) but with some variations. PC and PO samples show almost same inhibition closer enough to 1ppb. There is a significant difference between GC and GO. GC show more inhibition than GO and the opposite trend is seen in the case of IC and IO. IO is responsible for more inhibition than IC.

Same procedure was implemented to test the extracts with other solvents like hexane and toluene. With acetone and acetonitrile we experience a very poor response of detector which is not measueable (Fig 74). The activity of the extracts with the solvent hexane can be seen in Figure 78 and the resultant inhibition percent corresponding to each analyte is shown in Figure 79.

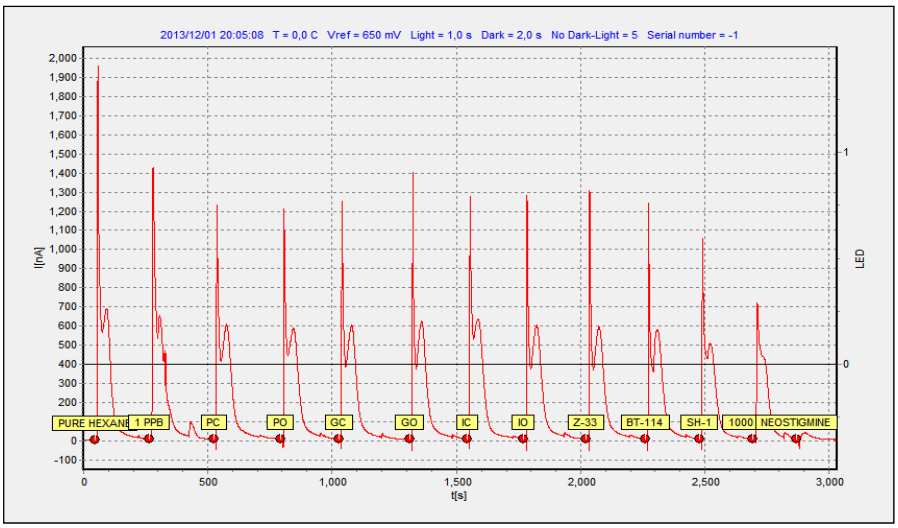


Figure 78: Amperometric response of all cotton samples extracted with hexane

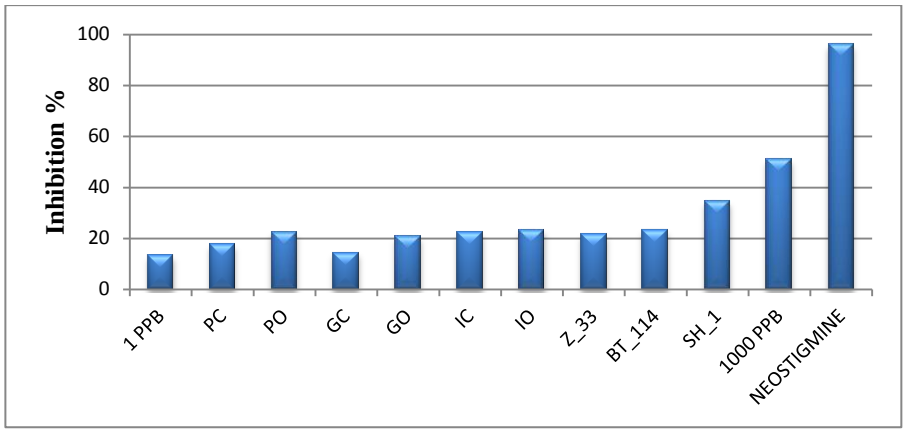


Figure 79: AChE-Inhibition caused by all cotton samples extracted with hexane

In case of hexane, more or less same trend has been observed. All of our extracts show the inhibition % (on average of < 40). There is some minor variations observed between classical and organic cotton samples. PO samples show more inhibition than PC. There is a some difference between GC and GO. GO show more inhibition than GC where as IC and IO show almost the same inhibition.

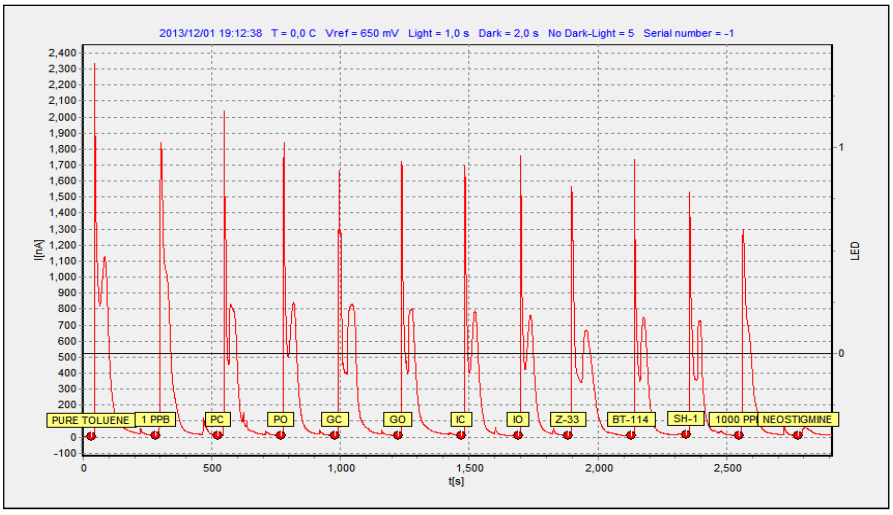


Figure 80: Amperometric response of all cotton samples extracted with toluene

The activity of the extracts with the solvent toluene can be seen in Figure 80 and the resultant inhibition percent corresponding to each analyte is shown in Figure 81.

In case of toluene, same trend has been observed as in hexane. All of our extracts show the inhibition % (on average of < 40) except SH-1 which shows slight more inhibition. The only difference is that IC shows more inhibition than IO.

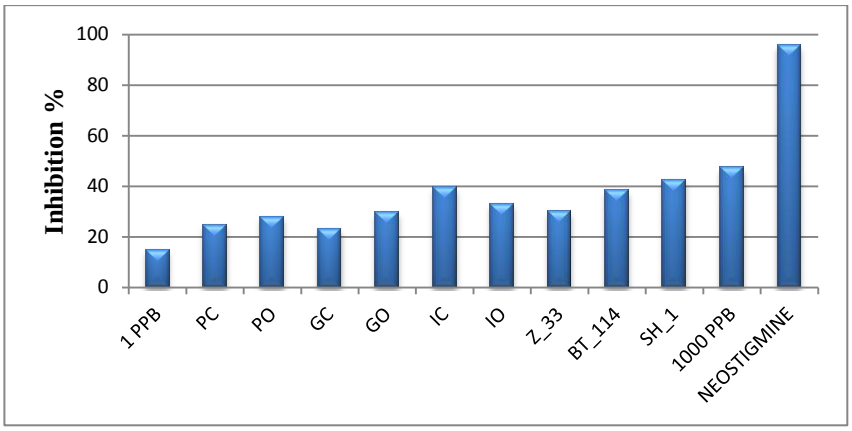


Figure 81: AChE-Inhibition caused by all cotton samples extracted with toluene

A summary of all the above mentioned experiments along with the solvents used is shown in Figure 82. It can be observed from this figure that there are some minor variations of using different solvents for extraction but as a whole, there is no significant difference of inhibition percent of AChE with respect to each individual sample.

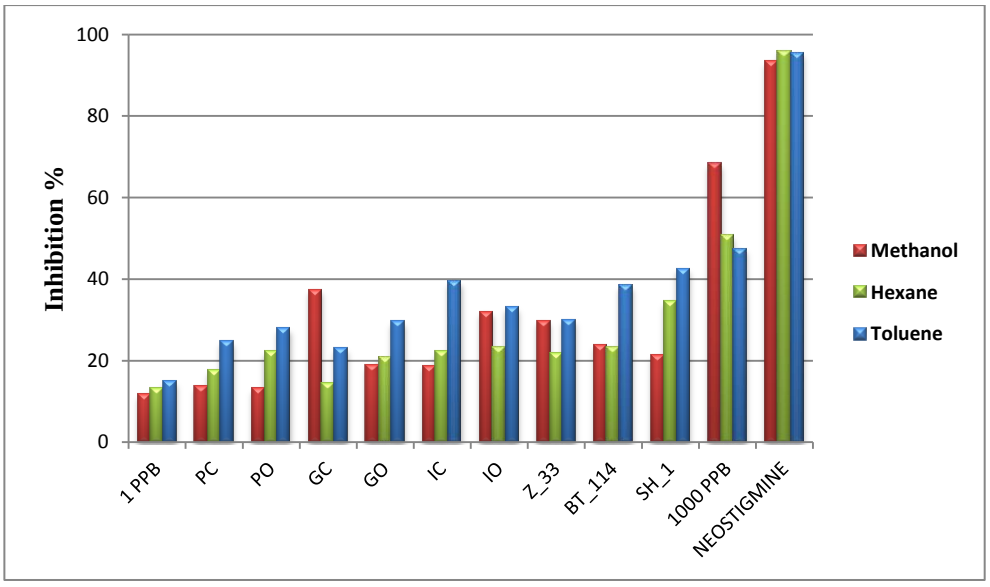


Figure 82: Summary of AChE-Inhibition caused by all cotton samples with all solvents

Also the difference of inhibition between classical and organic cotton samples is also not substantial. We can conclude that there may be possibility of the presence of AChE inhibitors in almost all our samples without any descrimination.

4.2 Life cycle assessment with Algae Growth Analyzer (AGA)

Single celled microalgae are among the most productive autotrophic organisms in nature due to their high photosynthetic efficiencies and the lack of heterotrophic tissues [156]. Algae possess a number of distinct physical and ecological features and their ability to proliferate over a wide range of environmental conditions reflects their diversity [17, 18]. Thus, inhibition of photosynthetic performance could also be used as a tool to evaluate the presence of pollutants [16].

Algae Growth Analyzer was used for the measurement of inhibition of photosynthetic activity of Algae. Green Algae of the family Scenedesmaceae and Genus SCENEDESMUS was arranged by Bvt technologies, Czech Republic.

All the resulted extracts from cotton samples (GC, GO, PC, PO, IC, IO) were arranged. Calibration of the device was done with 1 gm Na_2SO_4 and 5 ml Distilled water to consume all the oxygen inside the glass cell repeatedly for three times. Then it was washed with distilled water for three times.

All the resulted extracts were extracted by the solvent, Acetonitrile. We found some negative impact of this solvent on the growth of algae in the initial testing so as to ignore this impact of solvent and for achieving unbiased results; this solvent was evaporated completely at room temperature. 2 ml of all these extracts were put in petri dishes, separately, the solvent was evaporated and then the pure extracts were treated directly with 5 ml algae samples in petri dishes. We allowed them to cultivate for one hour and then the samples were tested by Algae Growth Analyzer.

All the above mentioned extracts were analyzed by AGA for a duration of 30 minutes each. With the help of miniature Oxygen electrode, we have obtained the oxygen production activity of the algae in presence of the extracts by recording the oxygen produced in medium. The initial amperometric response can be seen in Figure 83 which was then analyzed to get final results.

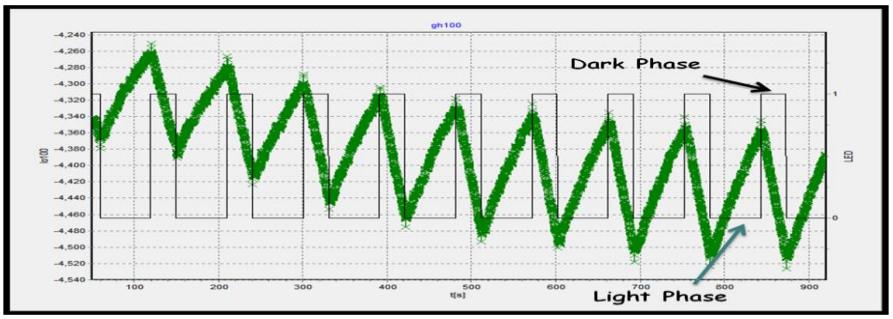


Figure 83: Initail Amperometric response

The results of Giza Cotton are shown in Figure 84. There are the differences in the oxygen production but in each case the addition of extract increases the production of oxygen.

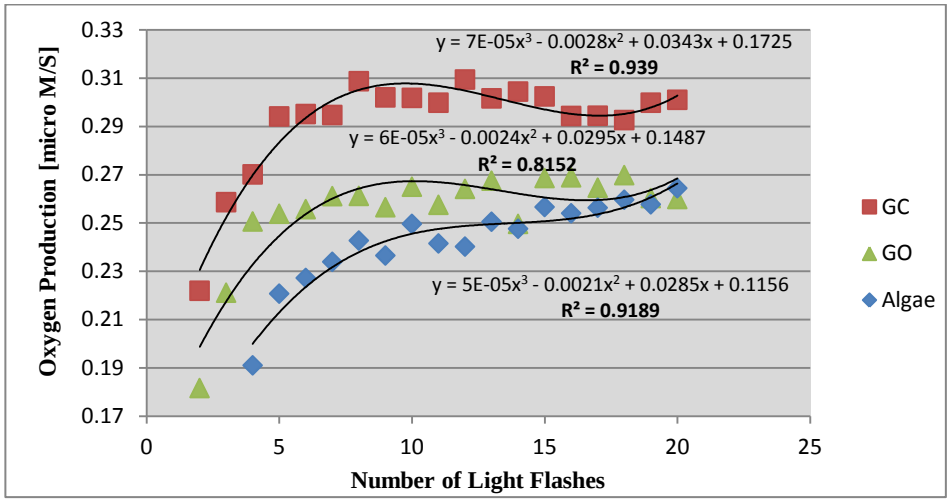


Figure 84: Comparison of GC & GO samples

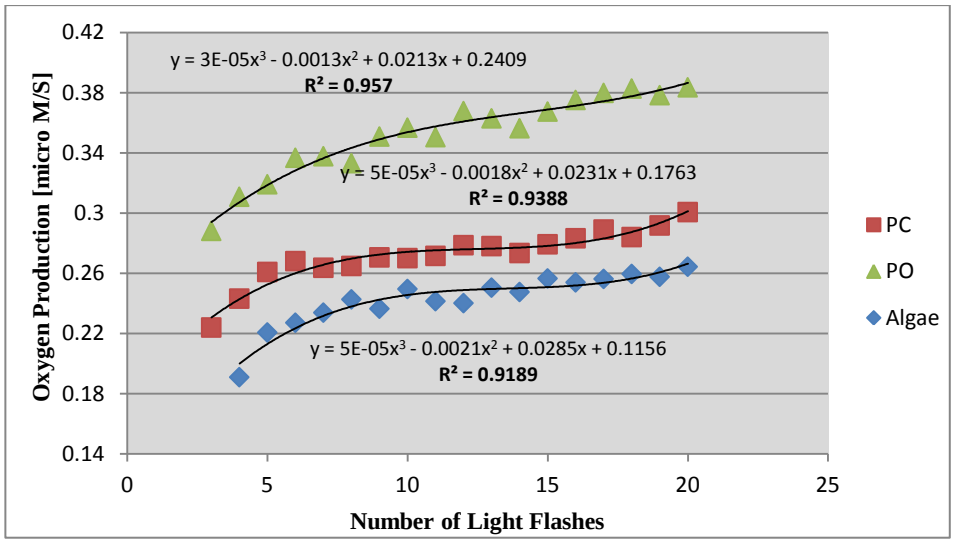


Figure 85: Comparison of PC & PO samples

Where as if we compare the classical and organic cotton, the stimulating agents in classical cotton are more and this is the reason of increase of oxygen production. Also it may be the possibility that the hazardous compounds in classical cotton are less than the organic one. However comparing the Pakistani classical and organic cotton (Fig 85), the stimulating agents in organic cotton are more and this is the cause of their high effect. Also it may be the possibility that the hazardous compounds in organic cotton are less than the classical one.

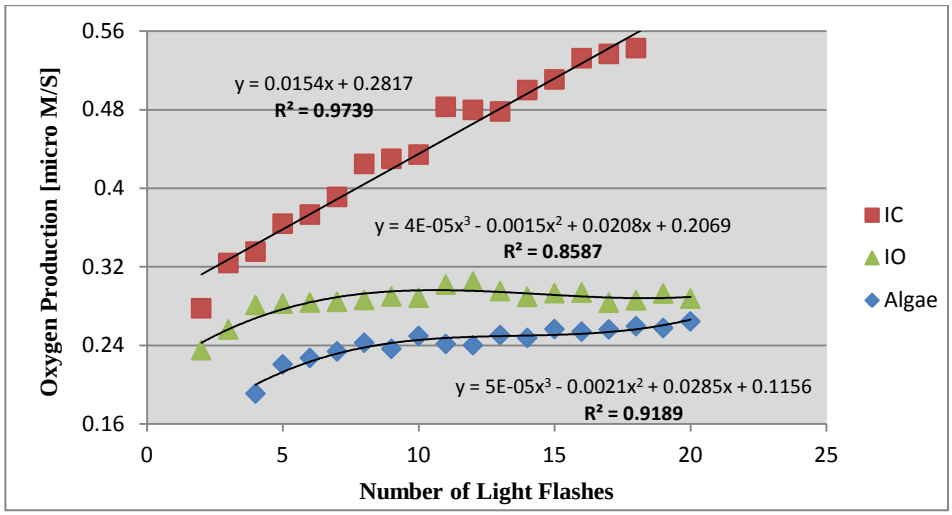


Figure 86: Comparison of IC and IO samples

The results of Indian cotton are shown in the Figure 86. It is quite visible that there is a significant difference in the oxygen production. Classical cotton shows higher production of oxygen in this case. Organic cotton extracts in this case may have some contaminants and pollutants which hinder in the streamline of oxygen production by the algae.

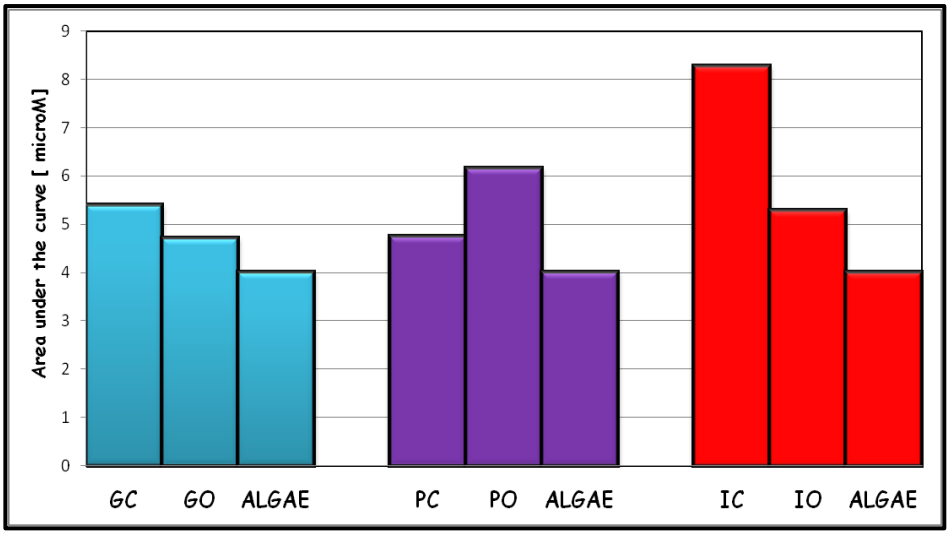


Figure 87: Comparison of area under the curve for all samples

The area under the curve for all the discussed samples is analyzed in Figure 87. Here we can see that there is a minor difference in case of Giza samples indicating less contaminants in GC as compared to GO. There is a major difference which can be seen in case of PC and PO samples where the PO samples contain less contaminants as compared to PC. In the last case there is also a noteable difference which indicates less harmful contaminants in case of IC as compared to IO samples.

4.3 Evaluation of Cytotoxicity

Moreover the both varieties of Egyptian Giza cotton samples were tested by Accredited Testing laboratory No. L 1540, Faculty of medicine, Masaryk University under the standard method CSN EN ISO 10993-5, Article 8.2, for cytotoxicity. It is a basic method providing the information about cytotoxicity. It is about the determination of cell growth activity of the population, by the number of cells growing in the sample extract and its dilutions. MG 63 cells were used for testing and all the extracts were held for 5 days at 37 °C followed by shaking at 60 rpm.

Cultures were incubated in a thermostat at 37°C ± -2 °C. Before starting the test the morphology of cultures, was verified in the microscope. The test was performed in the original extract (V1) and in dilutions of 1/2 (V1), 1/4 (V2), 1/8 (V3), 1/16 (V4). Every day the number of cells were counted. After the incubation period the cytotoxic effects were determined which are as follows:

0 Non cytotoxic – The number of living cell in sample is 80-100% of the number of cells in the negative control

1 Slightly cytotoxic – The number of living cell in sample is 70-80% of the cells in the negative control

2 Medium cytotoxic – The number of living cell in sample is 50-70% of the cells in the negative control

3 Highly toxic – The number of living cell in sample is under 50% of the cells in the negative control

4 Negative control: Medium extracted without sample (NK).

5 Positive control: The medium, in which the extracted sample is copper (PK).

The summary of number of cells after each day with different dilutions for normal cotton is shown in Table 15 and for organic cotton in Table 16, respectively. The growth curves for Giza Normal & Organic Cotton at different concentration levels have been plotted in Figure 88.

Table 15: Giza Classical Cotton Summary of Results

Time (Hrs)	Total No of Cells					
	V1	V2	V4	V8	PK	NK
0	20000	20000	20000	20000	20000	20000
24	22500	27500	22500	32500	20000	32500
48	60000	97500	87500	97500	27500	90000
72	183750	180000	195000	198750	47500	183750
96	292500	352500	345000	315000	67500	315000

Table 16: Giza Oragnic Cotton Summary of Results

Time (Hrs)	Total No of Cells					
	V1	V2	V4	V8	PK	NK
0	20000	20000	20000	20000	20000	20000
24	17500	20000	22500	22500	20000	20000
48	45000	50000	67500	65000	27500	30000
72	137500	142500	140000	112500	47500	102500
96	236250	281250	270000	225000	67500	213750

The decrease in number of cells can only be seen in the case of PK (Positive Control) inwhich the cells are interacted with copper. All the other cases with different dilutions show a stable increase of the cells during the whole period of testing.

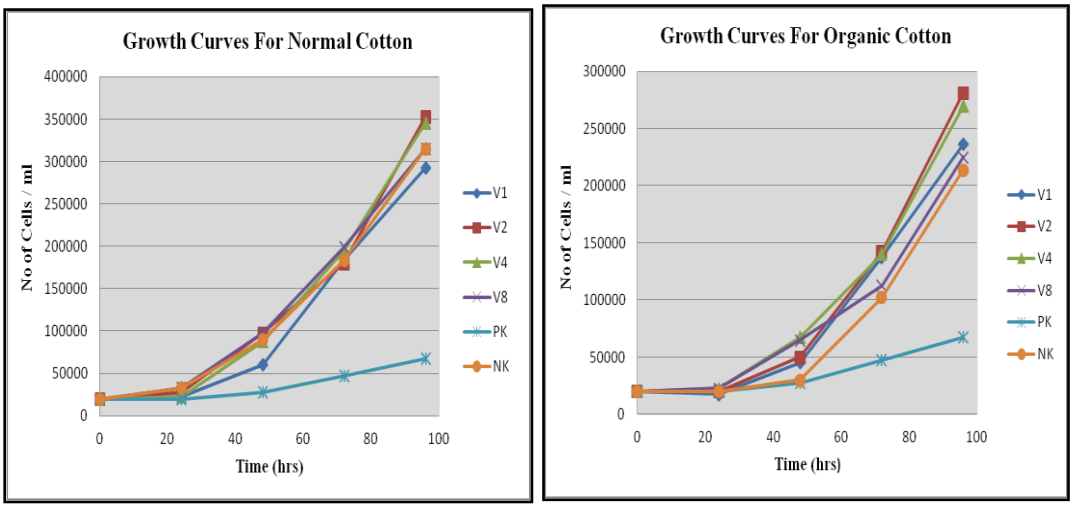


Figure 88: Growth curves for Normal & Organic Cotton at different concentration levels

The summary of above mentioned results is shown in Figure 89 and none of the cotton fibers (Normal and Organic) show any cytotoxicity as visible in the following graph.

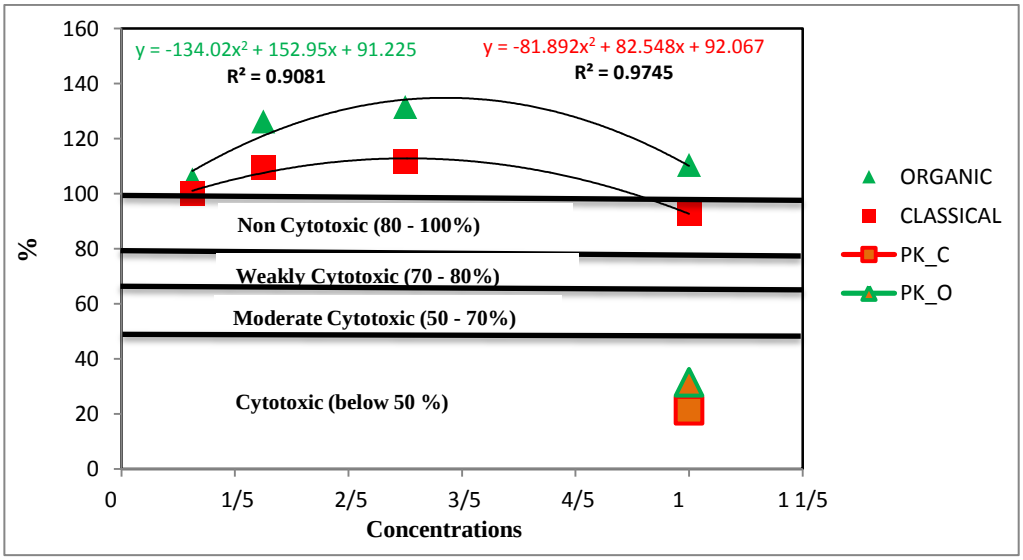


Figure 89: Comparison of the Normal and Organic Cotton regarding cytotoxicity

4.4 Conclusion

A rapid, sensitive and low cost method based on AChE-inhibition utilizing biosensor has been developed for the identification of residual pesticides. It can be seen throughout the testing that the enzyme inhibition is a complicated mechanism. All the variables involved in AChE inhibition activity have been studied and optimized such as enzyme & substrate concentrations, buffer, pH and incubation time. Each of these variables has a significant role in this mechanism. Suitable calibration curves were obtained by preparing 5 standard concentration levels of Mix 155 along with Neostigmine as standard inhibitor and analyzed in order of increasing concentration. The values of RSD of inhibition % for 5 repetitions are found to be in a range of 1.51 – 34.45. The detection limit is found to be below 1 ppb.

The method is utilized for real cotton samples extracted with different solvents (methanol, hexane, toluene). We are able not only to estimate the inhibition % of each individual sample but also we can compare this inhibition with the standard control points. The speciality of this method is that all the samples along with the control points can be tested in one run, The total time utilized for one complete test was approximately 50 ~ 55 minutes.

However in case of Algae testing, we can see that there is measurable interaction between cotton samples and algae which can be observed according to the results of our experiments but we are not able to find out some convincing results. The variation in the behavior of different cotton samples has been observed but none of these samples show any harm to the algae rather the effect of extracts stimulated their behavior. More concentrated samples must be employed in future to see some more interesting facts effects of this interaction. On the other hand algal species vary widely in their response to toxic chemicals and deferential sensitivity of green algae to the compounds has been observed in some reports. Compared with other kinds of detection devices, this method is simple and fast but it is much more sensitive for pesticide determination.

Chapter 5: Quantitative Analysis

The most efficient approach to pesticide analysis involves the use of chromatographic methods. Gas chromatography-mass spectrometry (GC-MS) with electron ionization (EI) and the combination of liquid chromatography (LC) with tandem mass spectrometry (LC-MS/MS) were identified as techniques most often applied in multi-residue methods for pesticides by Alder et al. [6]. GC-MS (gas chromatography-mass spectrometry) and GC-tandem MS are used for volatile or volatilizable analytes, while LC-MS/MS (liquid chromatography with tandem MS) is aimed at determining more polar and less volatile compounds [138]. For GC-amenable volatile and semivolatile pesticides GC methods are still preferred over LC methods due to higher resolution and lower detection limits [157].

GC-MS/MS allows to perform two consecutive stages of mass fragmentation in which parent ions fragmenting into daughter ions are monitored. This substantially improves selectivity and sensitivity of the determination compared to single-stage MS thanks to elimination of isobaric interferences and reduction of the chemical noise. Employing either of these techniques at the final determinative step is one of the most distinctive trends in pesticide residue analysis and is considered as a practical way to get around difficulties in target analytes identification in the case of difficult food and feed matrices containing excessive amounts of potentially interfering substances [25]. Keeping in view the above mentioned advantages, Gas Chromatography coupled to Tandem Mass Spectrometry was used for quantitative analysis.

5.1 Method development utilizing GC-MS/MS

A multiresidue method for analysis of 76 pesticides with different physicochemical properties was developed. The method involves a rapid and small-scale extraction procedure of real cotton samples collected from different regions (Egypt, Pakistan & India) with five different solvents (Methanol, Acetonitrile, Acetone, Toluene, Hexane) from polar to non polar region, using Ultra Sound assisted Extraction (USE). Cryogenic Homogenization was being implemented for sample Pre-treatment. After final extraction and filtration the extracts were concentrated.

The pesticide residues were determined by gas chromatography with Tandem mass spectrometry (GC-MS/MS). 57 out of 76 pesticides were detected successfully by the method developed. Nineteen (19) pesticides could not be analyzed by GC-MS/MS using EI ionization, most often because of incompatibility with evaporation of the intact molecule in the GC injector. Confirmation of pesticide and quantitation was performed in selected-reaction monitoring mode (SRM). The limit of detection (LOD), the limit of quantitation (LOQ) and precision have been worked out based on the guidelines for analytical measurements.

5.2 Method validation and quality control

All the essential parameters which are necessary for the method validation have been taken into account in the light of the document SANCO/12495/2011 for 'Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed'[158] which is the latest version of Commission Directive 96/46/EC. The guidelines were also taken from the guidance document SANCO/825/00 'Guidance document on pesticide residue analytical methods' [159].

Moreover the document from Codex Alimentarius document 'Guidelines on Good Laboratory Practice in Pesticide Residue Analysis' has been also considered [160].

The combination of sample traceability, sample preparation and processing, pesticide standards, preparation and storage of stock standards & working standards, extraction and concentration, calibration for quantification, acceptability of analytical performance, confirmation of results, reporting of results and finally interpretation of these results makes it possible to present a suitable analytical method with the guidelines taken from the above mentioned official documents.

Trueness, Repeatability, Specificity, Limit of detection (LOD), Limit of determination (LOQ) and Applicability have been experimentally determined for each individual relevant representative analyte.

5.2.1 Preparation of standards

The stock solution of individual pesticide standards of 10µgmL^{-1} were prepared by dissolving the appropriate amounts of the analytical standards in the relevant solvent. Working standard solutions were prepared by taking 10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) for each standard pesticide mix, separately. The dilutions of the pesticide standards were made with the same solvent

which they originally contain. To ease the process, the pesticide Mix 3 and Mix 14 were mixed together as they contain the same solvent and same concentration of the compounds. All the four pesticide standards (Mix 155, Mix 17, Mix 18, Mix 3 &14) are abbreviated as (KF, KS, KZ, KT).

5.2.2 GC-MS/MS Conditions

Detection of all analytes used electron impact (EI) MS/MS. This combination was favoured because less interference occurs and it achieves a higher degree of selectivity. In most studies using EI-MS the electron energy was either 70 eV or between 30 - 40 eV. In this work the default value of 70 eV was used. The analyses were done at selective reaction monitoring (SRM). Helium was used as carrier gas at a flow rate of 1 mL/min. The detailed description of GC parameters is being shown in the Table 17.

Table 17: Description of the GC Parameters

Type of GC	Thermo Scientific: Trace 1310 Gas Chromatograph
Column	TG-SQC
Column Length	15 meter
Column Oven Temperature Gradient	50^{O}C, 5 min → 12^{O}C/min → 260^{O}C, 7,5 min; Total Time 30 min
Injector Volume	Liquid, Splitless mode, 1 ul
Injector Temperature	250^{O}C
Split Flow	10 ml/min
Split Time	0.10 min
Carrier Flow	1 ml/min
Auto sampler	CTC Combi Pal
Mass Detector	Triple Quadrupole , TSQ 8000

The following temperature program was adopted for all subsequent studies reported. Figure 90 explains the temperature gradient of oven throughout the analysis.

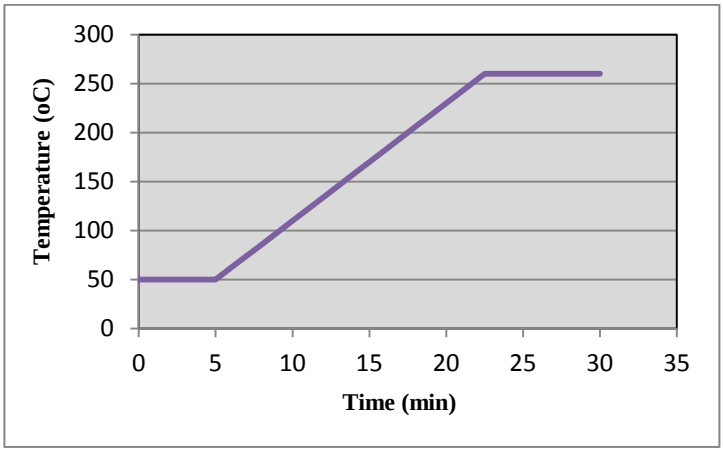


Figure 90: Description of column oven temperature gradient

5.2.3 Evaluation of Retention time

The working solution of $1 \mu g m L^{-1}$ of all the pesticide standard mixes (KF, KZ, KT, KS) was tested in EI-MS full scan mode for the typical mass range (35 to 500 amu). One of the resultant chromatograms has been shown in Figure 91 for Mix 18 (KZ). Evaluation of retention time is accomplished by comparing the probability of the presence of related ions evaluated by the related chromatograms and electron impact mass spectra of the analyte from the two built in database of libraries i.e. NIST and Mainlib.

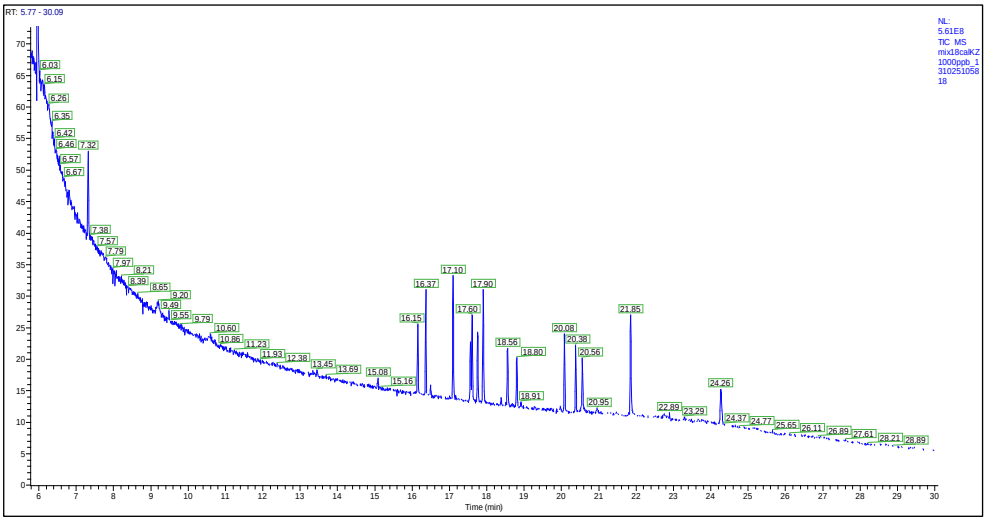


Figure 91: Gas Chromatogram for Pesticide Mix KZ

The NIST mass spectral database is a fully evaluated collection of electron ionization (EI) mass spectra with more than 70000 chemical compounds, compiled by the National Institute of Standards and Technology. It is the product of comprehensive evaluation and expansion of the world's most widely used mass spectral reference library. The other is Mainlib which contains more than 6000 library spectra.

Each peak of the chromatogram is analysed for each compound of the standard mix by comparing the mass to charge ratios of precursor and product ions with that of the two built in libraries. The criteria of acceptance have been set for probability of the presence of the analyte > 85% in both the libraries.

Figure 92 shows the mass to charge ratio for Primiphos-methyl attained from this above mentioned chromatogram. This mass spectrum is compared with the above mentioned databases. Figure 93 shows the resultant mass spectrum obtained from NIST database.

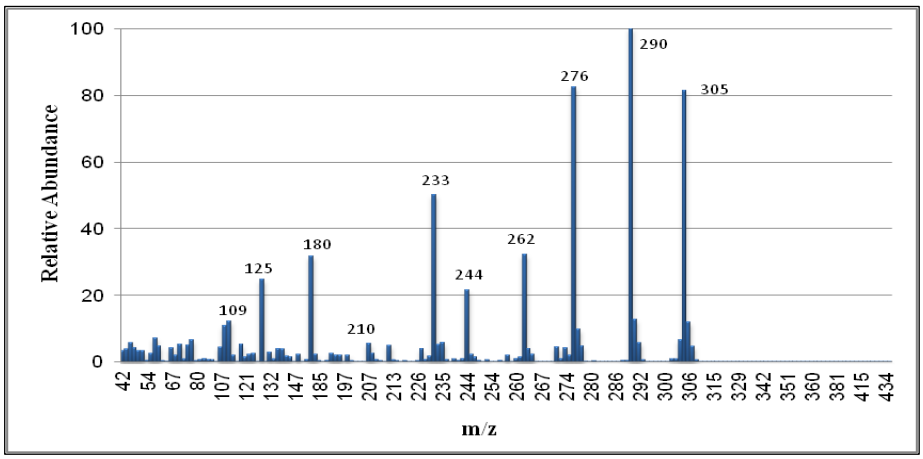


Figure 92: Mass to charge ratio for Primiphos-methyl

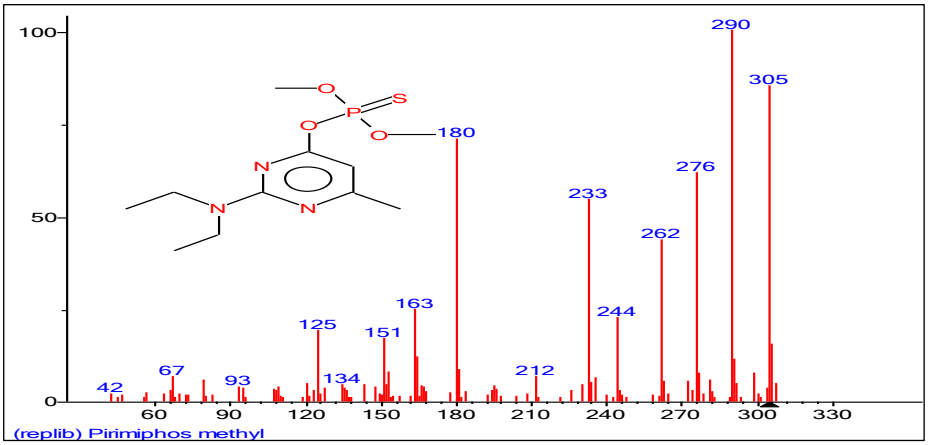


Figure 93: EI spectra, structure, and corresponding data for Primiphos-methyl from NIST database

The probability of presence of Primiphos-methyl in NIST is 97 % where as in Mainlib it was 97.03 %. These values are acceptable so the retention time evaluated for Primiphos methyl was 17.28 same as retention time of the corresponding peak in the main chromatogram.

Another example is of Dichlorvos, a compound from the same mix 18. Figure 94 shows the mass to charge ratio for Dichlorvos attained from related chromatogram. This mass spectrum is compared with the above mentioned databases. Figure 95 shows the resultant mass spectrum obtained from NIST database.

The probability of presence of Dichlorvos in NIST is 89.2 % where as in Mainlib it was 85.61 %. These values are acceptable so the retention time evaluated for Dichlorvos was 10.41 same as retention time of the corresponding peak in the main chromatogram. All the compounds of KZ were analyzed for the retention time in the same way and the summary of all the compounds of mix 18 has been shown in Table 21.

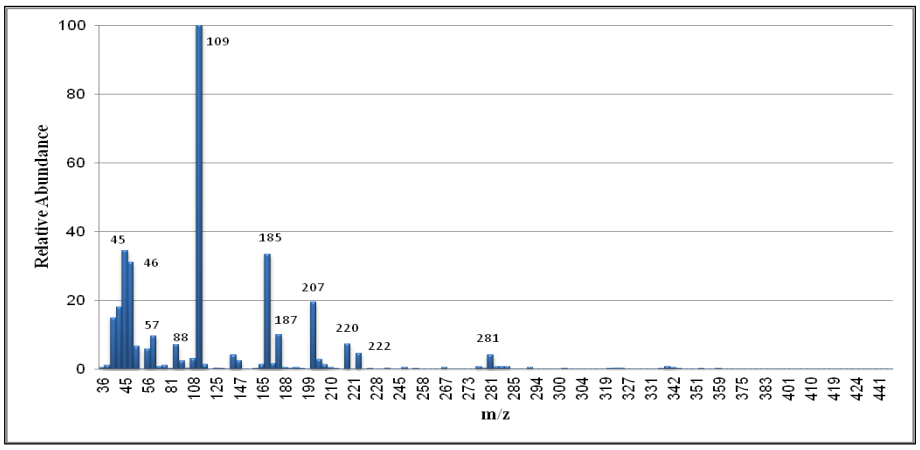


Figure 94: Mass to charge ratio for Dichlorvos

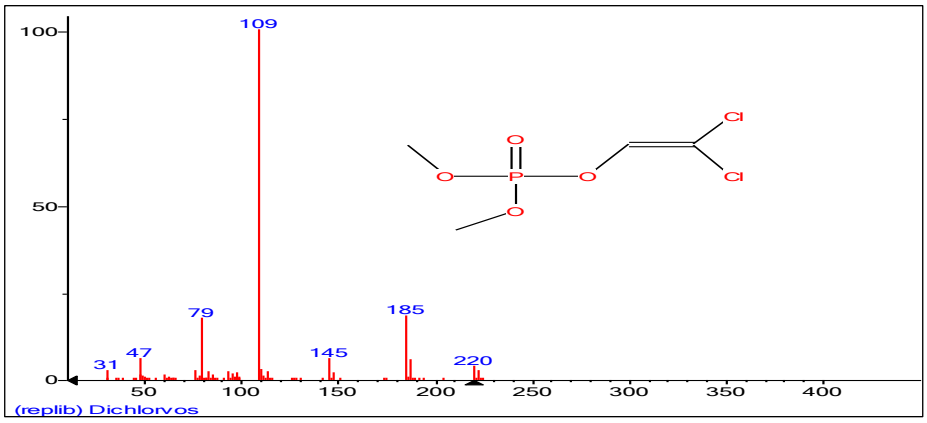


Figure 95: EI spectra, structure, and corresponding data for Dichlorvos from NIST database

The resultant chromatogram for Mix 3 & 14 (KT) has been shown in Figure 96. Evaluation of retention time is accomplished by comparing the probability of the presence of related ions evaluated by the related chromatograms and electron impact mass spectra of the analyte from the two built in database of libraries i.e. NIST and Mainlib.

Figure 97 shows the mass to charge ratio for Demeton-S-methyl-sulfone attained from the related chromatogram (Figure 96). This mass spectrum is compared with the built in databases. Figure 98 shows the resultant mass spectrum obtained from NIST database. The probability of presence of Demeton-S-methyl-sulfone in NIST is 96.3 % where as in Mainlib it was 95.77 %. These values are acceptable so the retention time evaluated for Demeton-S-methyl-sulfone was 17.08 same as the retention time of the corresponding peak in the main chromatogram. All the compounds of Mix 3 & 14 (KT) were analyzed for the retention time in the same way and the summary of all the compounds of Mix KT has been shown in Table 20.

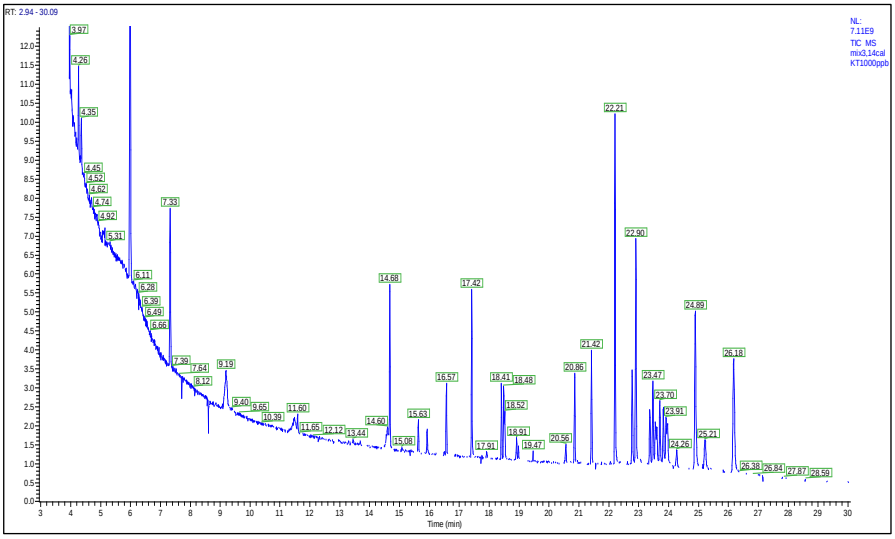


Figure 96: Gas Chromatogram for Pesticide Mix KT

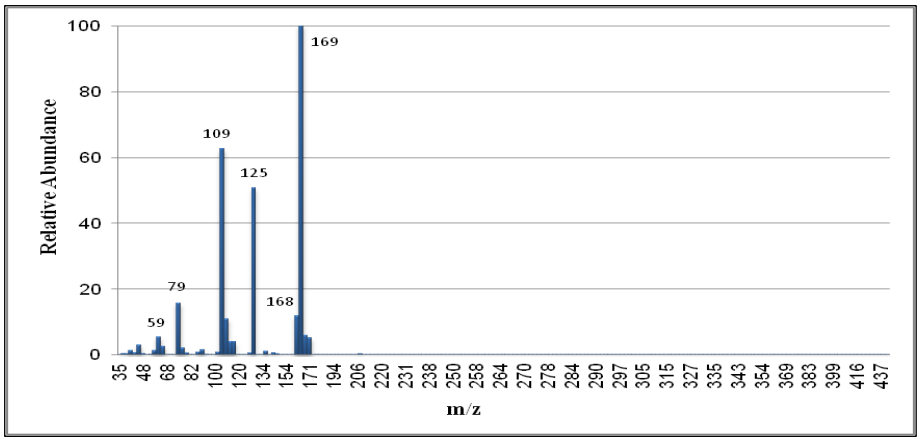


Figure 97: Mass to charge ratio for Demeton-S-methyl-sulfone

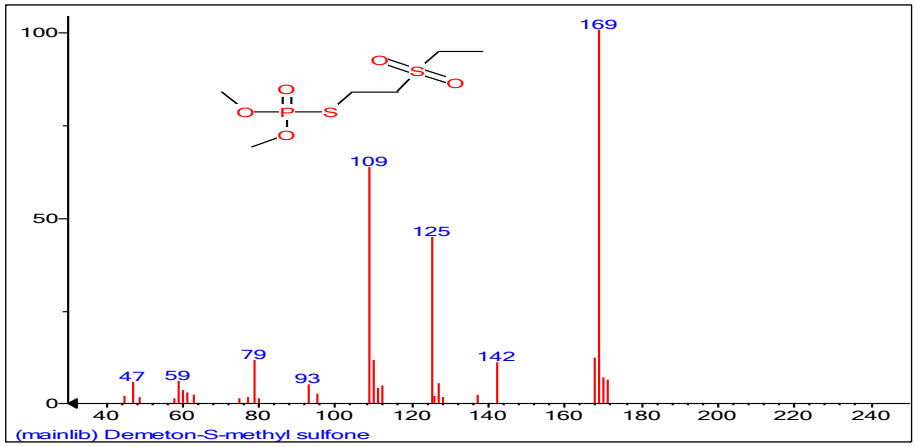


Figure 98: EI spectra and structure for Demeton-S-methyl-sulfone from NIST database

Pesticide standard Mix 155 (KF) and Mix 17 (KS) were analyzed for retention time in the same way as mentioned above and the detail of retention time of each analyte of these mixes have been shown in Table 18 and Table 19. After evaluating the retention

times of target compounds, we are able to update the pesticides in the Pesticide Compound Database (CDB) with the known retention times along with the precursor and product masses mostly used for these compounds.

Table 18: Retention time and precursor masses for KF

KF_MIX 155								
Analyte	**Retention Time (min)**	**Precursor Mass**	**Product Mass**	**Collision Energy**	**a**	**B**	**r^2**	**Concentration range (ng/mL)**
Thiometon	15.23	247	89	40	0.46	64.99	0.9998	0 - 50
Simazine	15.48	201	173	5	-15.01	3289.78	0.9966	0 - 100
Terbumeton	15.68	226	170	16	0.41	5.43	0.9962	0 - 50
Terbuthylazine	15.82	230	174	14	-0.72	119.16	0.991	0 - 100
Pirimicarb	16.48	238	166	10	-18.36	7523.22	0.9591	0 - 20
Terbutryn	17.21	242	186	25	0.76	63.47	0.9975	0 - 50
Pirimiphos-methyl	17.28	305	180	8	-41.70	2166.69	0.8893	0 - 20
Triadimefon	17.64	208	111	20	-28.54	2580.47	0.8992	0 - 20
Procymidone	18.38	283	96	10	-51.52	5367.01	0.9298	0 - 20
Vamidothion	18.59	145	87	10	0.54	-52.93	0.9997	0 - 500
Tetrachlorvinphos	18.65	329	109	38	0.30	109.22	0.9846	0 - 100
Profenofos	18.98	339	269	15	0.31	186.64	0.995	0 - 500
Triazophos	19.97	257	162	10	1.62	346.61	0.9978	0 - 500
Pyrazophos	22.06	374	222	35	0.0002	0.03	0.9955	0 - 1000

Table 19: Retention time and precursor masses for KS

KS_MIX 17								
Analyte	**Retention Time (min)**	**Precursor Mass**	**Product Mass**	**Collision Energy**	**a**	**b**	**r^2**	**Concentration range (ng/mL)**
1,2,4-Trichlorobenzene	9.27	180	109	20	4.17	11298	0.9965	0-50
1,2,4,5-Tetrachlorobenzene	11.27	214	108	30	311.09	6631.54	0.9992	0-5
Pentachlorobenzene	13.36	250	215	18	105.95	8390.98	0.9994	0-5
a-HCH	15.08	181	145	22	2.68	4890.11	0.9998	0-50
Hexachlorobenzene	15.2	284	214	30	2.19	5726.41	0.9998	0-50
b-HCH	15.58	219	145	28	-1.54	1288.59	0.9995	0-50
g-HCH	15.66	181	145	22	-0.90	4058.70	0.9995	0-50
Pentachloronitrobenzene	15.74	295	237	20	-0.49	863.49	0.9998	0-50
Heptachlor	16.83	274	237	20	-0.49	1150.30	0.9986	0-50
Aldrin	17.38	263	193	32	1.18	1483.24	0.9998	0-50
Heptachlorepoxid_trans	18.04	237	141	25	-0.15	231.98	0.9971	0-50
Endosulfan I	18.6	339	311	8	-0.009	2.32	0.9974	0-50
Dieldrin	18.99	277	206	18	0.02	440.26	0.9994	0-100
4,4'-DDE	19.01	246	176	30	-0.46	7444.48	0.9997	0-50
Endrin	19.32	279	243	10	-0.40	345.51	0.9996	0-50
Endosulfan II	19.47	195	159	14	1.56	443.07	0.9966	0-50
4,4'-DDD	19.65	235	165	22	-8.46	18067	0.9998	0-50
4,4'-DDT	20.2	235	165	22	-0.0002	0.10	0.9984	0-50
4,4'-Methoxychlor	21.09	227	141	35	29.51	835.03	0.9997	0-20

Table 20: Retention time and precursor masses for KT

KT_MIX 3 & 14								
Analyte	**Retention Time (min)**	**Precursor Mass**	**Product Mass**	**Collision Energy**	**a**	**b**	**r^2**	**Concentration range (ng/mL)**
Pentachlorophenol	15.59	267	167	20	-0.007	2.69	0.9625	0-200
Tefluthrin	16.25	177	137	15	9.482	4438.41	0.9998	0-50
Demeton-S-methyl-sulfone	17.08	169	79	20	0.744	165.07	0.9993	0-250
Dicofol (kelthane)	17.58	250	139	24	0.327	993.47	0.9964	0-20
Pendimethalin (penoxaline)	18.07	252	191	8	7.454	511.77	0.9976	0-20
Tolyfluanide	18.15	238	91	35	3.542	1343.63	0.9988	0-20
Captan	18.18	264	236	6	0.565	30.35	0.9998	0-500
Vamidothion	18.59	145	87	10	0.524	27.70	0.9997	0-400
Tetrachlorvinphos	18.65	329	109	38	2.690	188.08	0.9997	0-100
Captafol	20.51	313	114	14	0.072	0.87	0.9939	0-100
Permethrin	22.53	183	168	20	0.891	720.09	0.9981	0-100
Cyfluthrin	23.13	226	206	5	0.158	86.29	0.9966	0-250
Cypermethrin	23.4	181	152	22	0.620	348.78	0.9982	0-250
Fenvalerate	24.58	167	125	5	0.203	340.30	0.9899	0-250
Deltamethrin	25.72	253	172	8	0.020	0.08	0.9646	0-100

Table 21: Retention time and precursor masses for KZ

KZ_MIX 18								
Analyte	**Retention Time (min)**	**Precursor Mass**	**Product Mass**	**Collision Energy**	**a**	**b**	**r2**	**Concentration range (ng/mL)**
Dichlorvos	10.41	185	93	12	-25.68	6100.56	0.9995	0 - 100
Fonofos (dyfonate)	15.81	137	81	15	-20.73	4265.30	0.9995	0 - 100
Diazinon	16.04	304	162	8	-1.002	234.01	0.9976	0 - 100
Chlorpyrifos-methyl	16.76	286	93	23	-18.04	3524.75	0.9993	0 - 100
Pirimiphos-methyl	17.28	305	180	8	-4.38	1044.79	0.9992	0 - 100
Malathion	17.42	127	99	8	-56.38	10976.00	0.9992	0 - 100
Chlorfenvinphos	18.05	269	161	15	-0.88	224.80	0.9988	0 - 100
Methidathion	18.46	145	58	15	-21.76	4341.42	0.9991	0 - 100
Ethion	19.74	231	129	23	-23.48	4654.85	0.9992	0 - 100
Carbophenothion	20.04	342	157	10	-3.96	747.70	0.9990	0 - 100
Phosalone	21.50	182	138	8	-9.74	1960.57	0.9990	0 - 100

5.2.4 Calibration curves

For each pesticide standard mixes (KF, KS, KT, KZ), 10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) were prepared and analyzed in order of increasing concentration including related running blanks. The dilutions of the pesticide standards were made with the same solvent which they originally contain. Calibration curves were constructed by plotting concentration of each pesticide versus GC response (peak area).

The calibration curves of both external and internal standards of some analytes from each mix of KF, KS, KT & KZ can be seen in Figure 99, 100, 101 & 102, respectively.

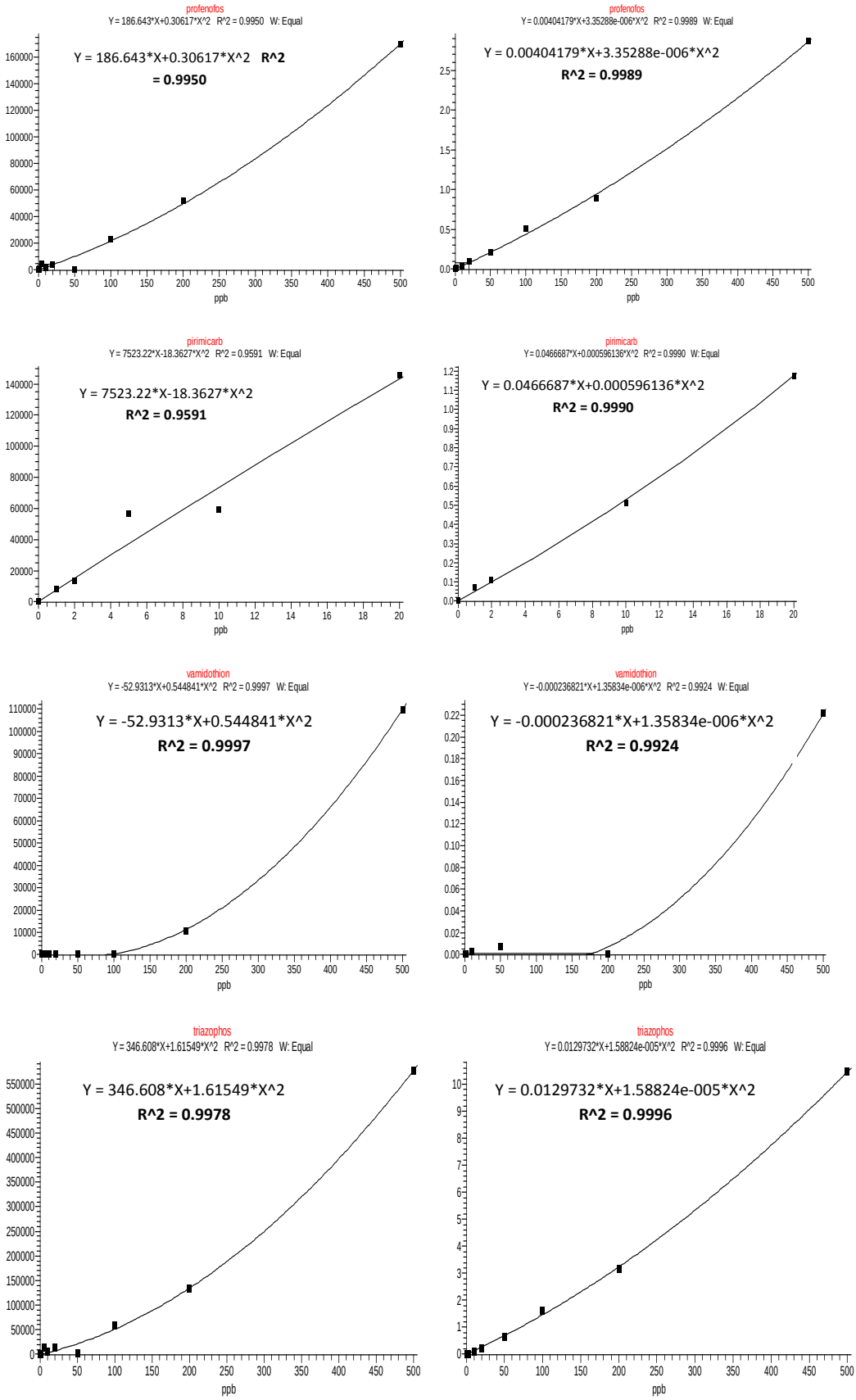


Figure 99: Calibration curves of KF with ESTD (Left) & ISTD (Right)

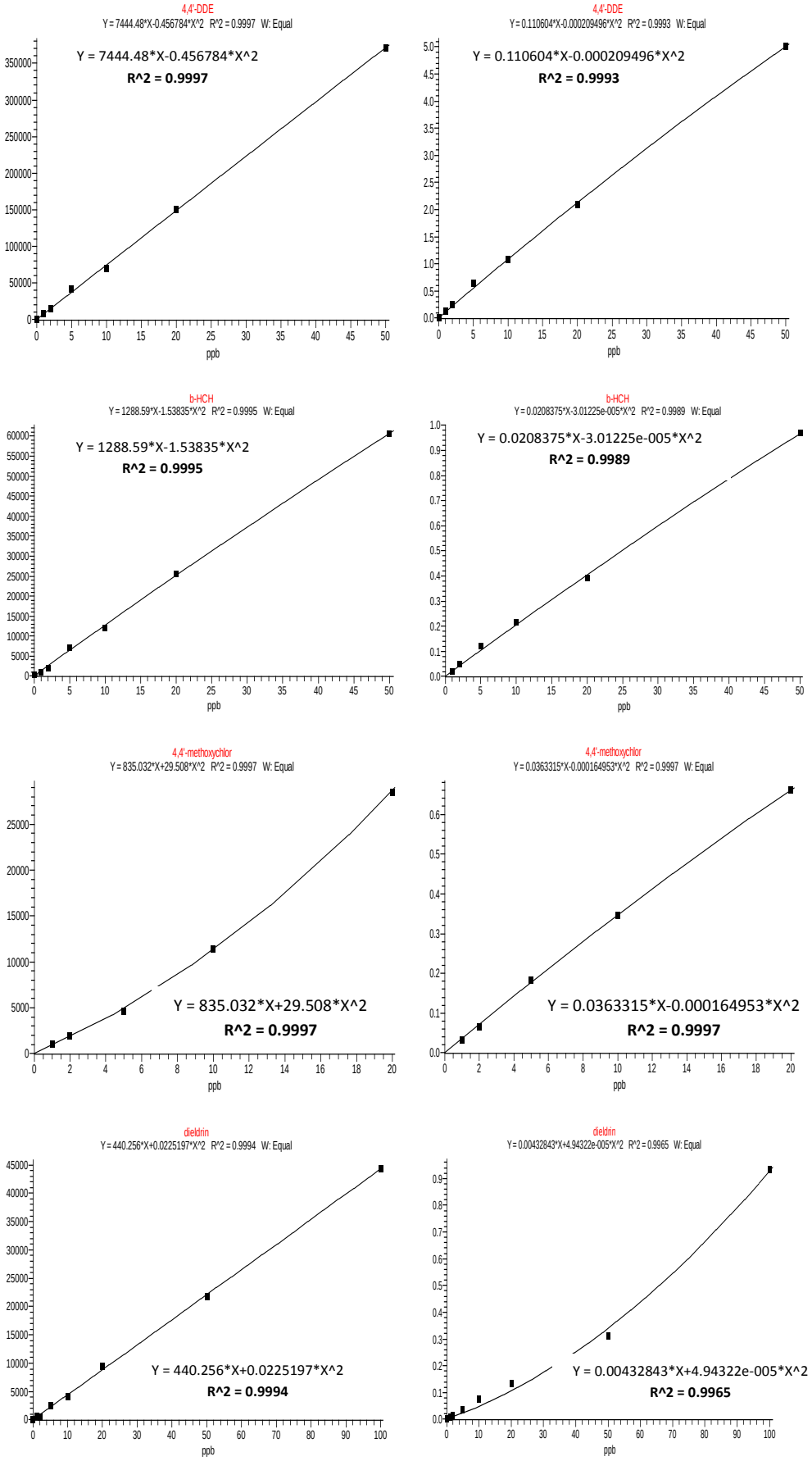


Figure 100: Calibration curves of KS with ESTD (Left) & ISTD (Right)

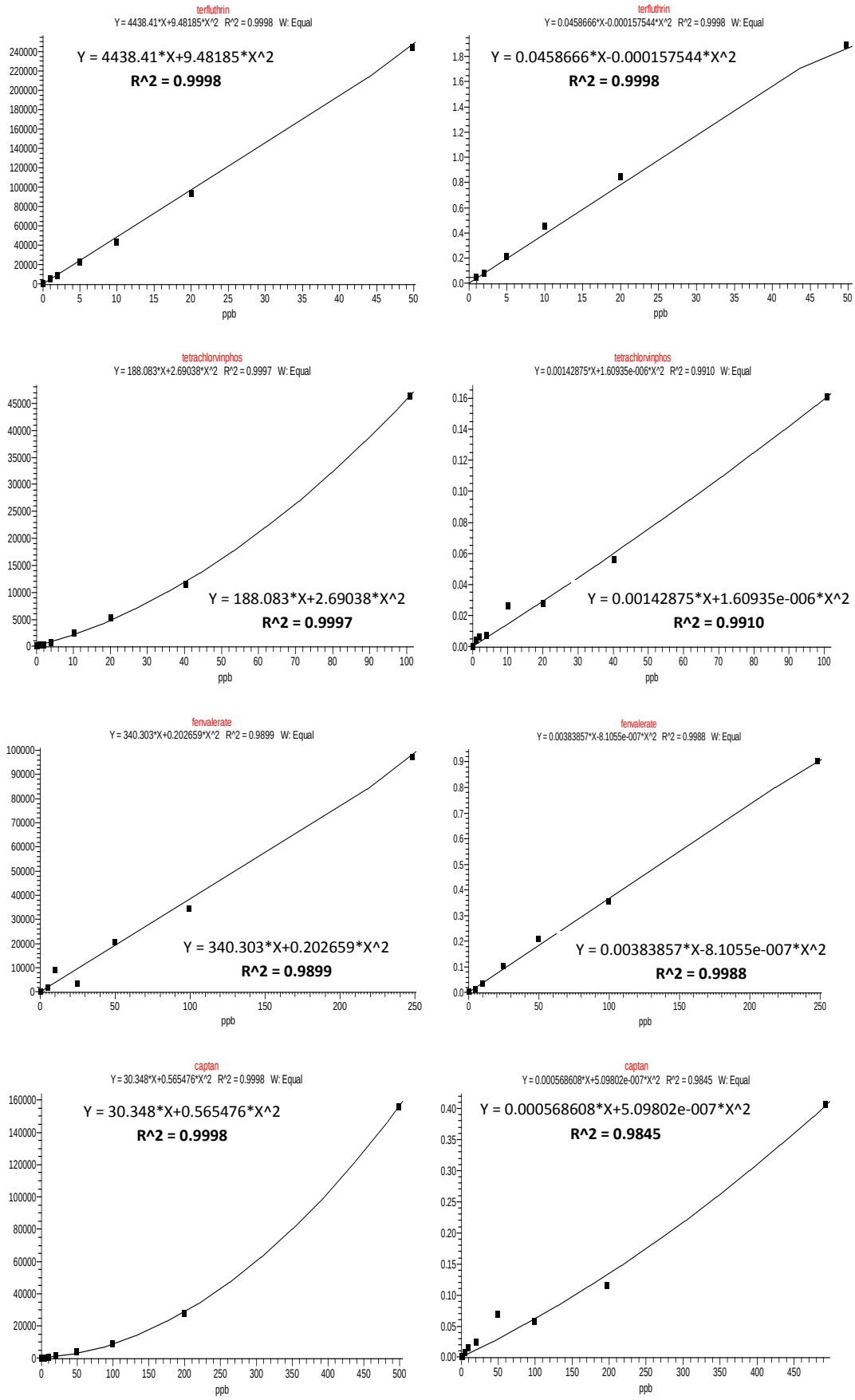


Figure 101: Calibration curves of KT with ESTD (Left) & ISTD (Right)

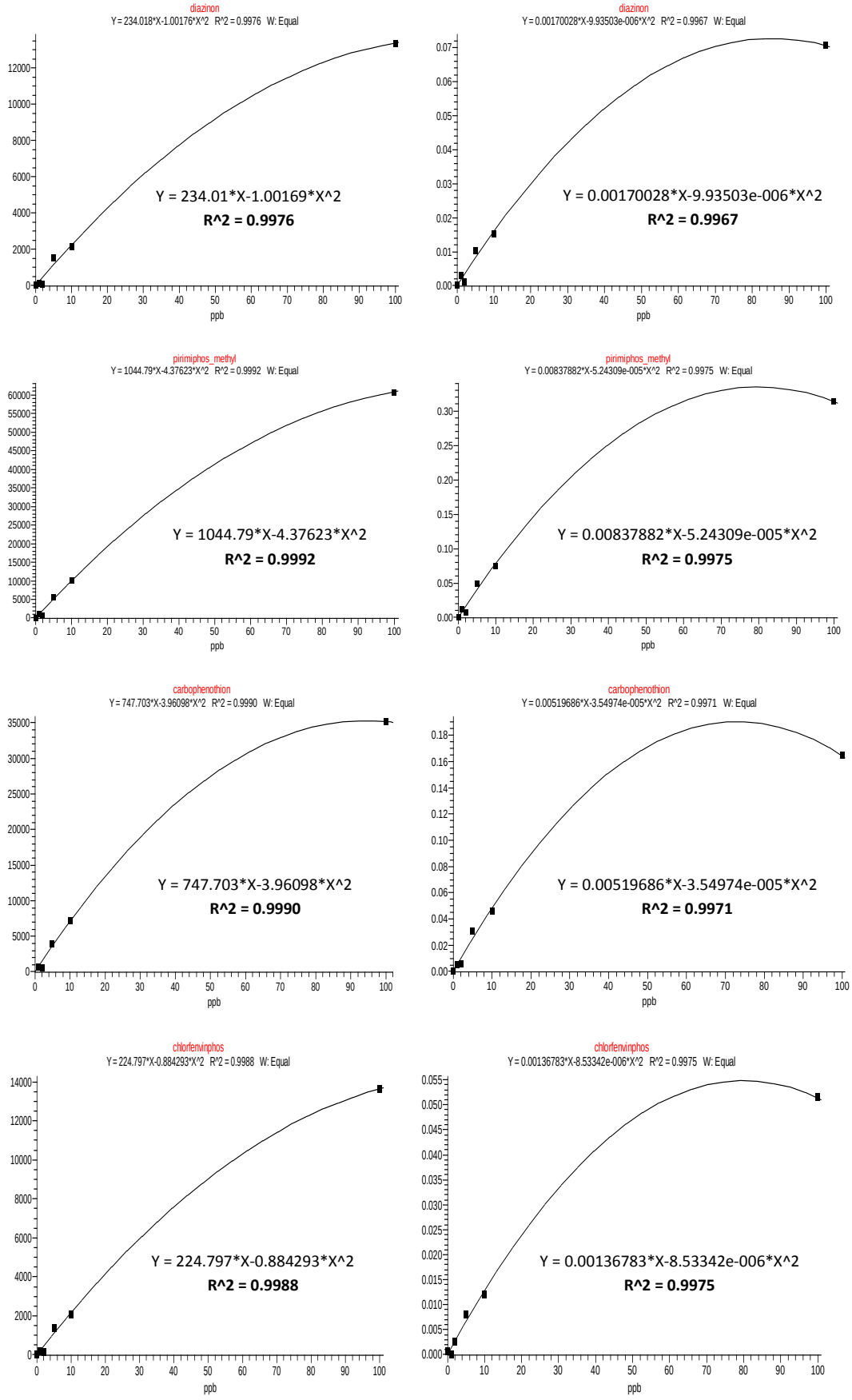


Figure 102: Calibration curves of KZ with ESTD (Left) & ISTD (Right)

For all analytes tested within a concentration range of 1–1000 ng/mL, the GC response was quadratic with excellent regression coefficients ($r^2 > 0.99$) as can be seen for KS & KZ in Table 19 & Table 21, with the exception of primicarb (0.9591), Primiphos-methyl (0.8893), triadimefon (0.8992), procymidone (0.9298), tetrachlorvinphos (0.9846) for KF (Table 18), and pentachlorophenol (0.9625), fenvalerate (0.9899), deltamethrin (0.9646) for KT (Table 20).

5.2.5 Accuracy and precision of developed method

The recovery, accuracy, and precision of the developed method were determined at the minimum concentration level i.e. 1 ng/mL for all mixes except KT (mix 3&14) for which it has been measured at 2 ng/mL. Each concentration contained ten replicates, although five replicates are recommended by [158]. Precision was calculated by using the relative standard deviation (R.S.D.). Accuracy was calculated by the following equation [161].

$$Accuracy = \frac{mean\ measured\ concentration}{nominal\ concentration} \times 100$$

According to the guidance document SANCO/12495/2011 of European Commission [158], the mean recovery should be in the range of 70–120% where as repeatability which is estimated by the relative standard deviation (RSD) of recoveries, should be ≤ 20% per commodity. According to Codex Guidelines 2003 the acceptable range of recoveries should be in between 60-120 % with a RSD value of 30 % [160].
High accuracy, good precision, and good reproducibility for all analytes of the standard pesticide mixes were achieved at the tested concentrations. There was a difference in precision and accuracy among different analytes; Captan has the highest accuracy 0f 120% (Table 24) where as 4,4´-DDT has a minimum precision value of 0.93 (Table 23).

The range of recoveries for all analytes have been varied between 81- 120 % where RSD values lied between 0.93 - 14.16 %. The accuracy and precision results for all these analyses are within the acceptable range as prescribed by [158 & 160].

5.2.6 Determination of LOD and LOQ

The limit of detection (LOD) is the minimum concentration of the analyte that can reliably be detected with a specified level of confidence. There are a number of approaches which can be used to evaluate the LOD. The LOD can be evaluated by obtaining the standard deviation of results obtained from replicate analysis of a sample containing only a small amount of the analyte.

Ideally, 6–10 replicate results for this sample, taken through the whole analytical procedure, should be obtained. The limit is calculated by multiplying the standard deviation by a suitable factor. The multiplying factor is based on statistical reasoning and is specified so that the risk of false positives (wrongly declaring the analyte to be present) and false negatives (wrongly declaring the analyte to be absent) is kept to an acceptable level (a 5% probability is usually specified for both types of error) [162].

A linear calibration graph between GC responses versus initial 5 concentration levels was constructed for which the slope has been determined. The limit of detection (LOD) was then calculated with the following equation:

$$LOD = \frac{3.3 * s}{m}$$

where *s* is the standard deviation of the 10 replicate measurements of the lowest concentration level. The variable *m* represents the slope of the calibration graph including blanks [104].

The limit of quantitation (LOQ) is the lowest concentration of analyte that can be determined with an acceptable level of uncertainty. A value of 10s is frequently used (where s is the standard deviation of the results from replicate measurements of the lowest concentration level). The aim is to identify the concentration below which the measurement uncertainty becomes unacceptable. The value of 10s provides a reasonable estimate for many test methods [162].

For all analytes tested within a concentration range of 0-10 ng/mL, the GC response was linear with excellent regression coefficients ($r^2 > 0.99$) with a few exceptions.

The Precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and regression coefficient for pesticide mix KF, KS, KT & KZ has been shown in Table 22-

25, respectively. The LODs for KF are in the range of 0.17 - 9.84 ng/mL, for KS 0.10 - 7.87 ng/mL, for KT 0.20 – 6.69 ng/mL and for KZ it is from 0.08 – 0.83 ng/mL. Whereas the LOQs for KF are in the range of 0.56 – 32.79 ng/mL, for KS 0.33 – 26.23 ng/mL, for KT 0.67 – 23.30 ng/mL and for KZ it is from 0.27 – 2.77 ng/mL.

Table 22: Precision, accuracy, LOD and LOQ description for KF

Analyte	Nominal Concentration (ng/mL)	Concentration measured (ng/mL)	Precision (R.S.D.)	Accuracy (%)	Limit of Detection (LOD) ng/mL	Limit of Quantitation (LOQ) ng/mL	r^2
Thiometon	1	1.02 ± 0.15	14.16	102	9.26	30.87	0.9996
Simazine	1	0.87 ± 0.03	3.52	87	1.14	3.79	0.9987
Terbumeton	1	0.89 ± 0.12	13.57	89	5.72	19.06	0.9997
Terbuthylazine	1	0.99 ± 0.01	1.04	99	0.24	0.81	0.9998
Pirimicarb	1	0.98 ± 0.05	5.32	98	0.50	1.67	0.9610
Terbutryn	1	1.14 ± 0.10	8.88	114	9.84	32.79	0.9388
Pirimiphos-methyl	1	0.99 ± 0.02	2.27	99	0.54	1.80	0.9992
Triadimefon	1	0.97 ± 0.05	5.46	97	0.30	1.00	0.9918
Procymidone	1	1.01 ± 0.02	1.82	101	0.17	0.56	0.9543
Vamidothion	1	0.90 ± 0.05	5.12	90	1.54	5.13	0.9784
Tetrachlorvinphos	1	0.98 ± 0.02	2.4	98	0.24	0.80	0.9019
Profenofos	1	0.96 ± 0.02	1.98	96	0.31	1.04	0.9998
Triazophos	1	0.99 ± 0.02	1.87	99	0.45	1.50	0.9733
Pyrazophos	1	1.05 ± 0.02	1.77	105	0.55	1.83	0.9998

Table 23: Precision, accuracy, LOD and LOQ description for KS

Analyte	Nominal Concentration (ng/mL)	Concentration measured (ng/mL)	Precision (R.S.D.)	Accuracy (%)	Limit of Detection (LOD) ng/mL	Limit of Quantitation (LOQ) ng/mL	r^2
1,2,4-Trichlorobenzene	1	1.19 ± 0.03	2.27	119	0.25	0.84	0.9840
1,2,4,5-Tetrachlorobenzene	1	1.05 ± 0.04	3.91	105	0.18	0.60	0.9977
Pentachlorobenzene	1	0.89 ± 0.04	4.09	89	0.10	0.33	0.9633
a-HCH	1	0.92 ± 0.02	2.32	92	0.63	2.11	0.9604
Hexachlorobenzene	1	1.03 ± 0.05	4.59	103	0.16	0.53	0.9882
b-HCH	1	0.88 ± 0.03	3.27	89	0.37	1.22	0.9998
g-HCH	1	1.13 ± 0.05	4.12	113	0.50	1.67	0.9902
Pentachloronitrobenzene	1	0.94 ± 0.04	3.90	94	0.26	0.87	0.9996
Heptachlor	1	1.02 ± 0.04	3.66	102	1.13	3.78	0.9967
Aldrin	1	0.94 ± 0.02	2.49	94	0.67	2.25	0.9980
Heptachlorepoxid_trans	1	1.11 ± 0.10	8.27	111	1.01	3.37	0.9838
Endosulfan I	1	0.89 ± 0.06	6.44	89	7.87	26.23	0.9888
Dieldrin	1	0.94 ± 0.02	1.56	94	0.58	1.93	0.9950
4,4'-DDE	1	1.02 ± 0.02	2.40	102	0.14	0.46	0.9919
Endrin	1	0.93 ± 0.07	7.71	93	0.61	2.02	0.9968
Endosulfan II	1	1.12 ± 0.03	2.98	112	0.36	1.21	0.9978
4,4'-DDD	1	0.98 ± 0.02	1.53	98	0.23	0.76	0.9973
4,4'-DDT	1	1.10 ± 0.01	0.93	110	0.82	2.75	0.9999
4,4'-Methoxychlor	1	1.03 ± 0.05	4.72	103	0.20	0.67	0.9976

Table 24: Precision, accuracy, LOD and LOQ description for KT

Analyte	Nominal Concentration (ng/mL)	Concentration measured (ng/mL)	Precision (R.S.D.)	Accuracy (%)	Limit of Detection (LOD) ng/mL	Limit of Quantitation (LOQ) ng/mL	r^2
Pentachlorophenol	2	2.08 ± 0.14	6.67	104	1.52	5.05	0.9998
Tefluthrin	2	1.89 ± 0.11	5.76	95	0.71	2.00	0.9873
Demeton-S-methyl-sulfone	2	1.99 ± 0.06	2.90	100	0.20	0.67	0.9949
Dicofol (kelthane)	2	1.77 ± 0.15	8.24	88	2.22	7.39	0.9918
Pendimethalin (penoxaline)	2	1.71 ± 0.09	5.2	85	1.89	6.29	0.9992
Tolyfluanide	2	2.29 ± 0.10	4.32	114	0.48	1.60	0.9808
Captan	2	2.40 ± 0.11	4.51	120	3.39	11.30	0.9734
Vamidothion	2	2.14 ± 0.05	2.17	107	1.20	4.01	0.9108
Tetrachlorvinphos	2	1.96 ± 0.06	3.19	98	0.24	0.82	0.9495
Captafol	2	2.17 ± 0.12	5.29	108	2.16	7.20	0.9575
Permethrin	2	1.75 ± 0.08	4.29	88	0.91	3.03	0.9962
Cyfluthrin	2	2.25 ± 0.10	4.29	112	0.90	3.00	0.9980
Cypermethrin	2	1.83 ± 0.03	1.75	91	0.60	2.01	0.9690
Fenvalerate	2	1.67 ± 0.08	4.45	84	0.25	0.84	0.9899
Deltamethrin	2	1.93 ± 0.13	6.57	97	6.99	23.30	0.9648

Table 25: Precision, accuracy, LOD and LOQ description for KZ

Analyte	Nominal Concentration (ng/mL)	Concentration measured (ng/mL)	Precision (R.S.D.)	Accuracy (%)	Limit of Detection (LOD) ng/mL	Limit of Quantitation (LOQ) ng/mL	r^2
Dichlorvos	1	1.03 ± 0.07	6.46	103	0.08	0.27	0.9999
Fonofos (dyfonate)	1	0.97 ± 0.11	11.14	97	0.83	2.77	0.9783
Diazinon	1	0.95 ± 0.04	3.84	95	0.70	2.34	0.9945
Chlorpyrifos-methyl	1	1.03 ± 0.03	2.57	103	0.18	0.60	0.9988
Pirimiphos-methyl	1	0.98 ± 0.08	8.15	98	0.35	1.17	0.9985
Malathion	1	1.12 ± 0.03	2.48	112	0.12	0.41	0.9011
Chlorfenvinphos	1	1.02 ± 0.08	7.98	102	0.53	1.78	0.9391
Methidathion	1	0.97 ± 0.05	4.64	97	0.14	0.47	0.9999
Ethion	1	0.90 ± 0.03	3.62	90	0.21	0.70	0.9999
Carbophenothion	1	0.99 ± 0.07	7.46	99	0.40	1.35	0.9987
Phosalone	1	0.81 ± 0.03	3.5	81	0.08	0.27	0.9977

5.3 Method application

Identification of target analytes is accomplished by comparing the retention time and electron impact mass spectra of the analytes to that of a standard analyzed under the same conditions. The quantitative interpretation of a gas chromatogram is based on peak area. The procedure for quantitation by the peak area depends upon the

measurement of the area of the peak of the compound from the extract solution to be analyzed and compared with the area of the peak measured for the compound from a standard solution, and from this comparison the amount of compound in the sample solution is calculated [163].

In order to evaluate the applicability of the developed method, real cotton samples extracted with different solvents (methanol, hexane, toluene, acetone & acetonitrile) were analyzed following the above mentioned methodology. Analysis of blanks provides information about the presence of contaminants so blank values must be reported and they should not be higher than 30% of the LOQ [159]. Method blanks for each pesticide standard were also analyzed. The criterion for acceptance of a peak is being realized by:

a) Matching Retention time
b) Peak shape
c) Peak within calibration points

Figure 103 & 104 shows a detailed sketch of the procedure being adopted for the acceptance of a peak.

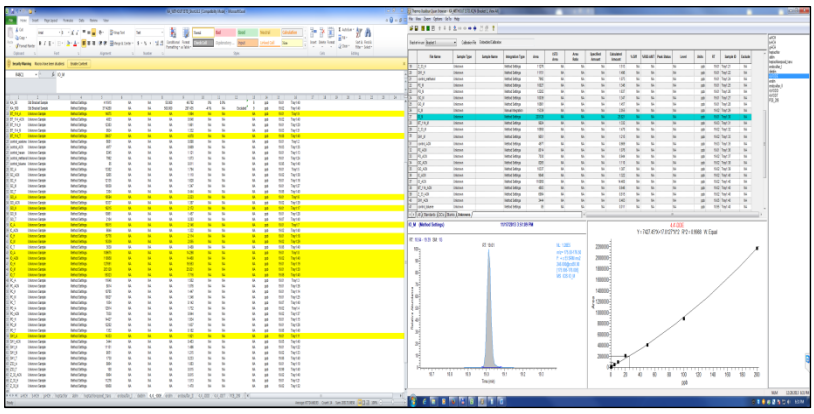


Figure 103: Quantitation of 4,4´-DDE (KS) in IC_H

Figure 103 shows the example of detection of 4,4´-DDE in only one sample i.e. indian cotton sample extracted in hexane (IC_H). On the left side there is a description of all the extracts to be analyzed along with peak area, retention time and the calculated amount of target compounds. On the right side there is a corresponding peak of the extract being monitored at that time (IC_H) and besides it there is a calibration curve of the standards. As we observed the same retention time of this peak from unknown

sample as RT of 4,4´-DDE, peak is in good shape and within calibration points so this peak has been accepted and the resultant calculated amount can be taken positively.

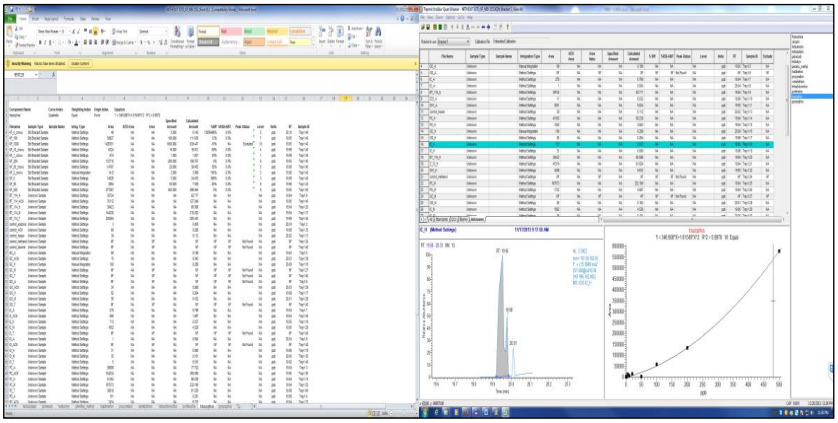


Figure 104: Quantitation of triazophos (KF) in IC_H

Figure 104 shows the detection of triazophos in Indian cotton sample extracted in hexane (IC_H). In this case RT was not matched and peak was also not in a proper shape so the peak has not been accepted. Also the calculated values below LOD have not been included.

To convert the peak areas to mass of analyte, whether from mass chromatograms, selected ion monitoring or selected reaction monitoring, the peak areas must be calibrated. The two main strategies are based on external and internal standards. These techniques have been used for quantitative analysis of residual pesticides on cotton samples.

5.3.1 Quantitation by external standardization

With external standards, the area of mass chromatogram is calibrated with a known amount of the standard analytes. For each pesticide standard mixes (KF, KS, KT, KZ), 10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) were prepared and analyzed in order of increasing concentration including related running blanks. Cotton samples extracted with different solvents (methanol, hexane, toluene, acetone & acetonitrile) were injected for analysis. The quantitation depends upon the measurement of the area of the peak of the compound from the extract solution to be analyzed and compared with the area of the peak measured for the standard concentration solution, and from this comparison the amount of compound in the sample solution is calculated.

The maximum residue limit (MRL) for cottonseed were also mentioned which are recommended by EU Pesticide Database [164] and Codex Alimentarius Commission database [165], as MRL values for cotton fibers have still not been established. The overall residual pesticides obtained by this method are summarized in Table 26.

Table 26: Description of pesticides detected with ESTD from KF

	Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Terbuthylazine (0.1)	GC_ACN	173	1.462	0.006	**Terbutryn**	GC_H	150	20.984*	0.084
	GC_H	129	1.091	0.004		GC_T	208	23.622*	0.094
	GO_A	201	1.708	0.007		IC_H	76	16.605*	0.066
	GO_T	125	1.055	0.004		IC_T	89	17.472*	0.070
	IC_H	313	2.669	0.011		PC_H	212	23.778*	0.095
	PC_A	276	2.351	0.009		PC_M	187	22.717*	0.091
	PC_ACN	266	2.260	0.009		PO_A	100	18.217*	0.073
	PO_ACN	501	4.318	0.017		PO_T	121	19.468*	0.078
	SH1_M	1102	9.829	0.039	**Tetrachlorvinphos**	BT114_A	57	0.524*	0.002
	Z33_T	1811	16.928	0.068		IO_ACN	73	0.664*	0.003
	Z33_M	1674	15.503	0.062		PO_ACN	32	0.297*	0.001
Profenofos (3)	GC_ACN	4263	22.042	0.088		PO_M	48	0.437*	0.002
	GC_H	3331	17.355	0.069		PO_T	47	0.431*	0.002
	GO_ACN	19768	92.024	0.368		Z33_T	113	1.029	0.004
	GO_H	20850	96.452	0.386		Z33_ACN	29	0.268*	0.001
	GO_M	17930	84.385	0.338	**Triazophos (0.2)**	BT114_A	39724	82.717	**0.331**
	IC_A	192	1.025*	0.004		BT114_H	38423	80.586	**0.322**
	IC_ACN	240	1.285	0.005		PC_A	36686	77.702	**0.311**
	IC_H	285	1.524	0.006		PC_H	43163	88.239	**0.353**
	IC_M	248	1.328	0.005		PC_T	38818	81.236	**0.325**
	IO_H	361	1.928	0.008		PO_ACN	2424	6.778	0.027
	IO_M	509	2.716	0.011		PO_M	1732	4.887	0.020
	SH1_ACN	1322	7.005	0.028		SH1_M	9299	24.117	0.096
	SH1_H	1231	6.525	0.026		Z33_ACN	73150	131.027	**0.524**
	SH1_M	1196	6.342	0.025		Z33_M	104084	168.290	**0.673**
	Z33_T	3084	16.097	0.064					

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

Terbuthylazine, Profenofos, Terbutryn, Tetrachlorvinphos & Triazophos from KF were found present in the cotton samples. In case of Triazophos, 7 samples out of ten exceed MRL. The worth mentioning point is that PC (Pakistani classical) cotton samples contain more amount of residual pesticides than PO samples. Most samples contain values greater than LOD but lower than LOQ e.g. in case of Tetrachlorvinphos and Terbutryn.

Table 27 represents the residues detected considering the KS which contains mostly organochlorine (OC) pesticides. The complete list of the pesticides detected from KS can be observed in Appendix 6A and 6B.

Table 27: Description of pesticides detected with ESTD from KS

	Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Aldrin (0.02)	BT114_A	25235	18.409	**0.074**	**4,4'-DDE (0.05)**	BT114_A	14875	1.998	0.008
	GC_A	15502	11.359	**0.045**		BT114_H	12383	1.664	0.007
	GO_A	22416	16.373	**0.065**		BT114_M	9924	1.333	0.005
	IO_A	19555	13.049	**0.052**		GC_ACN	8265	1.110	0.004
	IO_ACN	7170	4.815	0.019		GC_H	12135	1.630	0.007
	PC_A	16761	12.274	**0.049**		GC_M	10039	1.349	0.005
	PO_A	12708	9.324	**0.037**		GO_ACN	10337	1.389	0.006
	PO_ACN	6371	4.281	0.017		GO_H	16215	2.178	0.009
4,4'-Methoxychlor (0.01)	BT114_H	2400	2.630	**0.011**		GO_M	10861	1.459	0.006
	BT114_M	2288	2.516	**0.010**		IC_A	16015	2.152	0.009
	SH1_A	7810	7.412	**0.030**		IC_ACN	9846	1.323	0.005
	SH1_ACN	9790	8.915	**0.036**		IC_H	15308	2.057	0.008
	SH1_H	8825	8.195	**0.033**		IO_A	109675	14.746	**0.059**
	SH1_M	8180	7.700	**0.031**		IO_ACN	110958	14.918	0.060
	Z33_T	1034	1.188	0.005		IO_H	127691	17.171	**0.069**
	Z33_H	1327	1.509	0.006		PC_A	11646	1.564	0.006
	Z33_M	7604	7.249	**0.029**		PC_ACN	8014	1.077	0.004
a-HCH (0.02)	BT114_A	10309	3.713	0.015		PC_M	10027	1.347	0.005
	BT114_T	30595	6.235	**0.025**		PO_A	12914	1.735	0.007
	GO_A	15607	3.186	0.013		PO_ACN	7030	0.944	0.004
	GO_ACN	6593	2.378	0.010		PO_M	12202	1.639	0.007
	IC_A	10702	3.854	0.015		SH1_A	14333	1.925	0.008
	PC_A	8266	1.689*	0.007		SH1_H	11151	1.498	0.006
	PC_ACN	5071	1.036*	0.004		SH1_M	9051	1.216	0.005
	PO_ACN	5340	1.091*	0.004		Z33_ACN	6064	0.815	0.003
	Z33_T	72005	14.607	**0.058**		Z33_M	10958	1.472	0.006

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

4,4'-DDT, 4,4'-DDD, 4,4'-DDE, Aldrin, 1,2,4-Trichlorobenzene, a-HCH, b-HCH, g-HCH & 4,4'-Methoxychlor were detected in different cotton samples. In case of Aldrin and 4,4'-Methoxychlor, mostly samples exceed MRL. Aldrin is detected with exceeded MRL values in both samples of PC & PO and also GC & GO. Mostly samples containing 4,4'-DDT have the values >LOD but < LOQ (Appendix 6A). Only one sample IO (Indian organic cotton) contain 4, 4'-DDE with exceeded MRL. The residues of all other OC insecticides remained below MRL in all samples.

The residues obtained for KT are summarized in Table 28. Considering this mix, only Fenvalerate & Captan were detected with exceeded MRL values. Both samples of GC & GO contain the residues more than MRL being established for this pesticide. PC

samples with different solvents contain the residues of Captan with exceeded MRL. Mostly samples contain the residual values of Dicofol >LOD but < LOQ.

Table 28: Description of pesticides detected with ESTD from KT

	Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Pentachlorophenol	IC_A	74	29.139	0.117	**Tetrachlorvinphos**	IO_ACN	83	0.437*	0.002
	IC_H	86	35.399	0.142		PO_M	46	0.245*	0.001
Dicofol (kelthane) (0.1)	BT114_A	2777	2.793*	0.011		PO_T	47	0.250*	0.001
	BT114_H	2477	2.492*	0.010	**Fenvalerate (0.05)**	GC_ACN	10242	29.577	**0.118**
	BT114_M	2614	2.629*	0.011		GC_H	12617	36.291	**0.145**
	IO_A	5280	5.305*	0.021		GC_M	11307	32.595	**0.130**
	IO_ACN	6425	6.454*	0.026		GO_A	3639	10.626	0.043
	IO_H	8133	8.165	0.033		GO_H	7203	20.905	**0.084**
	PC_A	5051	5.076*	0.020	**Captan (0.02)**	PC_A	323	9.105*	**0.036**
	PC_H	2166	2.179*	0.009		PC_ACN	437	11.814	**0.047**
	PC_M	2332	2.345*	0.009		SH1_M	3862	60.054	**0.240**
	PO_A	3726	3.746*	0.015		Z33_H	388	10.657*	**0.043**
	PO_H	2683	2.698*	0.011		Z33_M	458	12.543	**0.050**
	PO_M	2700	2.716*	0.011	**Demeton-S-methyl-sulfone**	BT114_A	3020	16.996	0.068
	Z33_T	1578	1.588*	0.006		BT114_H	1829	10.574	0.042
	Z_33_H	2246	2.259*	0.009		GC_M	964	5.694	0.023
	Z_33_M	2310	2.323*	0.009		GO_M	1572	9.146	0.037
Vamidothion	BT114_A	14093	139.664	0.559		PO_ACN	1861	10.751	0.043
	BT114_H	10238	115.809	0.463		PO_M	5232	28.134	0.113
	IO_A	272	8.471	0.034		SH1_H	3650	20.261	0.081
	PC_A	279	8.664	0.035		SH1_M	2649	15.033	0.060
	PC_ACN	3157	55.555	0.222		Z33_ACN	5834	31.011	0.124
	Z33_T	21176	171.425	0.686		Z33_M	4028	21.668	0.087
	Z33_H	22586	182.834	0.731					

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

Table 29 represents the overall residues detected considering the mix KZ which contains mostly organophosphorous (OP) pesticides. Malathion, Fonofos, Chlorpyrifos methyl, Ethion & Phosalone were detected in different samples. Only the samples (BT114, SH1 & Z33) are found to have residual values of pesticides more than MRL as can be seen in the following table. All the other samples containing the residues are well below MRL.

Table 29: Description of pesticides detected with ESTD from KZ

	Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Malathion (0.02)	BT114_M	88038	8.382	**0.0335**	**Ethion (0.02)**	BT114_H	1787	0.385*	0.0015
	BT114_T	91536	8.731	**0.0349**		BT114_M	13573	2.960	0.0118
	GO_M	13795	1.265	0.0051		SH1_A	7205	1.560	0.0062
	IO_M	18640	1.713	0.0069		SH1_ACN	5598	1.210	0.0048
	PC_ACN	14711	1.350	0.0054		SH1_M	15069	3.292	0.0132
	PC_M	51338	4.795	0.0192		Z33_T	141581	37.516	**0.1501**
	PO_A	3862	0.352*	0.0014		Z33_ACN	66120	15.401	**0.0616**
	PO_ACN	9781	0.895*	0.0036		Z33_M	129889	33.599	**0.1344**
	SH1_A	34794	3.223	0.0129	**Phosalone (0.02)**	BT114_A	2844	1.461	0.0058
	SH1_M	88891	8.467	**0.0339**		BT114_ACN	3525	1.814	0.0073
	Z33_T	54800	5.128	**0.0205**		BT114_H	2107	1.080	0.0043
	Z33_M	109758	10.574	**0.0423**		SH1_A	7939	4.134	0.0165
Fonofos (0.1)	GC_ACN	13030	3.102	0.0124		SH1_ACN	9604	5.024	**0.0201**
	PO_ACN	7707	1.823*	0.0073		SH1_H	10630	5.577	**0.0223**
	SH1_A	21899	5.269	0.0211		SH1_M	7327	3.809	0.0152
	SH1_ACN	23645	5.702	0.0228		Z33_ACN	3455	1.778	0.0071
	SH1_H	13473	3.209	0.0128		Z33_H	1970	1.010	0.0040
Chlorpyrifos_methyl (0.05)	BT114_T	22998	6.759	0.0270					
	SH1_T	1740	0.495*	0.0020					
	Z33_T	30899	9.200	0.0368					

* Values >LOD but < LOQ
Analytes that exceed MRL are in **bold** type.

5.3.2 Quantitation by internal standard method

The strategy that gives the most accurate quantitative results is the use of internal standards, which are known amounts of compounds added to the sample before isolation of the analytes begins. After sample extraction and cleanup, only the ratio of response between the analyte and the internal standard must be measured. This ratio multiplied by the amount of the internal standard gives the amount of the analyte injected into the GC system. This can be converted to concentration using the correct dilution factors.

The best internal standards are chemically very similar to the analyte; thus, any losses of the analyte during the analytical procedure are duplicated by losses of the internal standard, so it is a self-correcting system. Depending on the relationship of the internal standard to the analyte, the precision and accuracy of most analyses are improved by at least a factor of 2 to 3 over external calibration [123].

The reliability of the results can be improved by evaluating the analysis with the aid of an internal standard. An advantage of using an internal standard for quantitation is that

inaccuracies during injection and alterations of detector response have hardly any influence on the analytical result. [163].

An internal standard (IS) is a chemical compound added to the sample test portion or sample extract in a known quantity, at a specified stage of the analysis, in order to check the correct execution of the analytical procedure. The IS should be chemically stable and typically show the same behaviour as of the target analyte [158].

Polychlorinated biphenyls (PCBs) have been widely used as complex mixtures in heat transfer fluids, dielectric fluids in capacitors and transformers and as additives in paints, pesticides, copy paper, adhesives and plastics. PCB 209 is used as an internal standard. An amount of 0.4 µg/mL was added homogeneously in all the cotton sample extracts along with method blanks and all calibration samples prior to the analysis. Figure 105 shows the detector response for five additions of PCB 209.

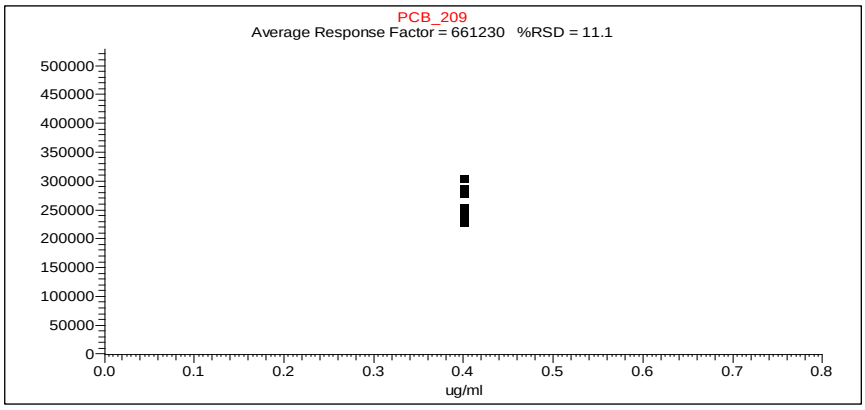


Figure 105: GC response for PCB 209 (ISTD)

The area ratio of response between the analyte and the internal standard is measured. The quantitation depends upon the measurement of the area of the peak of the compound from the extract solution to be analyzed and compared with the area of the peak measured for the internal standard and from this comparison the amount of compound in the sample solution is calculated.

The results obtained from this methodology are presented in Table 30 for KF. Profenofos, Terbutryn, Terbuthylazine, Triazophos & Tetrachlorvinphos were detected. The residues of all insecticides in KF remained below MRL in all samples with the exception of Z33_M having residue more than MRL.

Table 30: Description of pesticides detected with ISTD from KF

	Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Profenofos (3)	GC_ACN	0.018	4.328	0.017	**Terbuthylazine (0.1)**	GC_ACN	0.0014	1.018	0.004
	GC_H	0.014	3.458	0.014		GC_H	0.0004	0.277*	0.001
	GO_ACN	0.080	19.473	0.078		GO_T	0.0009	0.660*	0.003
	GO_H	0.088	21.510	0.086		PC_A	0.0015	1.036	0.004
	GO_M	0.090	21.796	0.087		PO_ACN	0.0012	0.927	0.004
	IC_ACN	0.003	0.683*	0.003		PO_M	0.0009	0.661*	0.003
	IC_H	0.002	0.438*	0.002	**Triazophos (0.2)**	BT114_A	0.187	14.165	0.057
	IC_M	0.002	0.451*	0.002		BT114_H	0.181	13.723	0.055
	IO_ACN	0.002	0.520*	0.002		PC_A	0.247	18.583	0.074
	IO_H	0.002	0.392*	0.002		PC_H	0.251	18.931	0.076
	SH1_ACN	0.007	1.742	0.007		PC_T	0.286	21.501	0.086
	SH1_H	0.006	1.522	0.006		PO_ACN	0.010	0.761*	0.003
	SH1_M	0.005	1.289	0.005		PO_M	0.007	0.547*	0.002
	Z33_T	0.012	3.029	0.012		SH1_M	0.053	4.073	0.016
Terbutryn	GC_H	0.00062	58.388	0.234		Z33_ACN	0.425	31.563	0.126
	GC_T	0.00053	54.818	0.219		Z33_M	0.751	54.256	**0.217**
	PO_T	0.00004	36.486	0.146	**Tetrachlorvinphos**	IO_ACN	0.001	0.265*	0.001

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

Table 31 represents the residues detected considering KS which contains mostly organochlorine (OC) pesticides. 4,4'-DDD, 4,4'-DDE, Aldrin, 1,2,4-Trichlorobenzene, a-HCH, b-HCH, g-HCH & 4,4'-Methoxychlor were detected in different cotton samples. The complete list of all analytes detected can be observed in Appendix 7.

In case of Aldrin, mostly samples exceed MRL. Aldrin is detected with exceeded MRL values in both samples of PC & PO (same is the case while using ESTD). Mostly samples containing 4,4'-DDD and 4,4'-DDE have the values >LOD but < LOQ so these values cannot be taken positively with confidence. Only one sample Z_33 contain a-HCH with exceeded MRL.

Although the residues of DDD and DDE were detected in almost all samples but the residues of these insecticides were remained below MRL.

Table 31: Description of pesticides detected with ISTD from KS

	Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Aldrin (0.02)	BT114_A	0.285	12.099	**0.048**	**4,4'-DDE (0.05)**	BT114_M	0.023	0.209*	0.001
	GC_ACN	0.068	2.857	0.011		GC_ACN	0.024	0.214*	0.001
	GO_A	0.280	11.921	**0.048**		GC_M	0.020	0.178*	0.001
	IO_A	0.260	11.045	**0.044**		GO_ACN	0.029	0.261*	0.001
	IO_ACN	0.076	3.183	0.013		GO_H	0.024	0.216*	0.001
	PC_A	0.298	12.671	**0.051**		GO_M	0.027	0.246*	0.001
	PO_A	0.460	13.277	**0.053**		IC_A	0.026	0.236*	0.001
	PO_ACN	0.083	3.450	0.014		IC_ACN	0.045	0.408*	0.002
4,4'-Methoxychlor (0.01)	BT114_H	0.008	0.223*	0.001		IC_H	0.030	0.267*	0.001
	BT114_M	0.033	0.921	0.004		IC_M	0.052	0.468	0.002
	SH1_A	0.039	1.073	0.004		IC_T	0.023	0.204*	0.001
	SH1_ACN	0.049	1.349	0.005		IO_A	0.591	5.395	0.022
	SH1_H	0.042	1.166	0.005		IO_ACN	0.692	6.337	0.025
	SH1_M	0.063	1.740	0.007		IO_H	0.611	5.587	0.022
	Z33_T	0.013	0.361*	0.001		IO_M	0.763	6.987	0.028
	Z33_M	0.059	1.636	0.007		PC_A	0.017	0.156*	0.001
a-HCH (0.02)	BT114_A	0.065	1.308*	0.005		PC_ACN	0.018	0.159*	0.001
	BT114_T	0.116	1.488*	0.006		PO_A	0.024	0.213*	0.001
	GO_A	0.112	1.430*	0.006		PO_ACN	0.020	0.185*	0.001
	GO_ACN	0.032	0.635*	0.003		SH1_M	0.017	0.153*	0.001
	IC_A	0.070	1.409*	0.006		Z33_ACN	0.016	0.148*	0.001
	PC_A	0.076	0.969*	0.004		Z33_M	0.017	0.153*	0.001
	Z33_T	0.291	5.850	**0.023**					

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

The residual pesticides obtained for KT are summarized in Table 32. Considering this mix, only Dicofol, Fenvalerate & Captan were detected with exceeded MRL values. GC contains the residues of Fenvalerate more than MRL being established for this pesticide. PC sample contains the residues of Captan with exceeded MRL. Mostly samples contain the residual values of Dicofol >LOD but < LOQ.

Table 32: Description of pesticides detected with ISTD from KT

	Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Dicofol (kelthane) (0.1)	IO_ACN	0.036	31.326	**0.125**	**Demeton-S-methyl-sulfone**	BT114_A	1.359	187.494	0.750
	IO_H	0.031	26.763	**0.107**		BT114_H	1.301	180.393	0.722
	IO_M	0.049	43.173	**0.173**		GC_M	0.066	10.559	0.042
	PC_ACN	0.002	1.829*	0.007		PO_ACN	5.369	575.086	2.300
	PO_A	0.006	4.948*	0.020		PO_M	2.783	343.902	1.376
	Z33_T	0.003	2.867*	0.011		SH1_H	0.079	12.577	0.050
	Z33_H	0.003	2.767*	0.011		SH1_M	0.093	14.773	0.059
	Z33_M	0.003	2.378*	0.010		Z33_ACN	0.016	2.559	0.010
Captan (0.02)	PC_ACN	0.017	28.784	**0.115**		Z33_H	0.015	2.345	0.009
	SH1_M	0.024	41.103	**0.164**	**Vamidothion**	PC_A	0.0001	7.590	0.030
	Z33_M	0.027	45.391	**0.182**		PC_ACN	0.0159	82.572	0.330
Fenvalerate (0.05)	GC_ACN	0.042	10.918	0.044		Z33_H	0.1254	253.197	1.013
	GC_H	0.062	16.300	**0.065**	**Tetrachlorvinphos**	IO_ACN	0.001	0.641*	0.003

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

Table 33: Description of pesticides detected with ISTD from KZ

	Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
Malathion (0.02)	BT114_M	0.244	3.773	0.015	**Ethion (0.02)**	BT114_H	0.01	0.368*	0.001
	GO_M	0.039	0.585	0.002		BT114_M	0.022	0.825	0.003
	BT114_T	0.1	1.521	0.006		SH1_ACN	0.05	1.911	0.008
	IO_M	0.044	0.662	0.003		SH1_M	0.074	2.842	0.011
	PC_ACN	0.073	1.107	0.004		Z33_T	0.675	32.587	**0.130**
	P0_A	0.050	0.754	0.003		Z33_ACN	0.352	14.697	**0.059**
	SH1_M	0.364	5.712	**0.023**	**Phosalone (0.02)**	BT114_ACN	0.016	1.136	0.005
	Z33_T	0.114	1.741	0.007		BT114_H	0.011	0.755	0.003
	Z33_M	0.103	1.567	0.006		SH1_ACN	0.062	4.413	0.018
Fonofos (0.1)	GC_ACN	0.109	4.664	0.019		SH1_H	0.045	3.171	0.013
	SH1_ACN	0.088	3.749	0.015		SH1_M	0.045	3.184	0.013
	SH1_H	0.043	1.791	0.007		Z33_H	0.015	1.036	0.004
Chlorpyrifos_methyl (0.05)	BT114_T	0.086	4.102	0.016					
	SH1_T	0.007	0.341*	0.001					
	Z33_T	0.122	5.869	0.023					

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

Table 33 represents the list of residues detected considering the KZ which contains mostly organophosphorous (OP) pesticides. Malathion & Ethion were detected in different samples. Only the samples (SH1 & Z33) are found to have residual values of pesticides more than MRL as can be seen in the following table. All the other samples containing the residues are well below MRL.

5.4 Conclusion

A multiresidue method for analysis of 76 pesticides with different physico-chemical properties has been developed for quantitative determination. The pesticide residues were determined by gas chromatography with Tandem mass spectrometry (GC-MS/MS). 57 out of 76 pesticides were detected successfully by the method developed. Nineteen (19) pesticides could not be analyzed by GC-MS/MS using EI ionization, most often because of incompatibility with evaporation of the intact molecule in the GC injector. Confirmation of pesticide and quantitation was performed in selected-reaction monitoring mode (SRM). The range of recoveries for all analytes have been varied between 81- 120 % where RSD values lied between 0.93 - 14.16 %. The accuracy and

precision results for all of these analyses have been found within the acceptable range as prescribed by [158 & 160].

The method was capable of detecting pesticides in real cotton samples. The GC-MS/MS method described in this work provides a reliable procedure for the determination of residual pesticides on cotton fibers. The procedure was proven to be effective, fast, sensitive and applicable to a wide range of pesticides. All validation criteria mentioned by European Commission document SANCO/12495/2011 for 'Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed' [158] were fulfilled. The method gave satisfactory analytical performance parameters for the most of the targeted pesticides and analysis of real samples proved its feasibility for the intended purpose.

Chapter 6: Summary

6.1 Conclusions drawn from the work

Owing to the introduction of a number of sophisticated technologies and instruments, tremendous improvements in the ability to analyze multiple pesticides for multiple classes in a variety of sample matrices have occurred in recent years. A growing number of techniques are available to the analytical chemist, and many strategies are possible to meet the purpose of analysis. In general, the use of the fewest analytical steps that provide reliable results in a rugged approach serves as the best overall approach to determining pesticide residues in food, environmental , and other types of samples. A host of strategies is available but practical concerns in the laboratory, such as time, budgets, available instruments, and personnel, limit the amount of effort and resources that can be devoted to the analysis. The thesis includes a study of three different techniques for the investigation of residual pesticides on cotton.

The aim of the work was to contribute to a research field which is moving toward the development of very wide range screening methods. A rapid, sensitive and low cost method based on AChE-inhibition utilizing biosensor has been developed for the identification of residual pesticides. It can be seen throughout the testing that the enzyme inhibition is a complicated mechanism. All the variables involved in AChE inhibition activity have been studied and optimized such as enzyme & substrate concentrations, buffer, pH and incubation time. Each of these variables has a significant role in this mechanism. Suitable calibration curves were obtained by preparing 5 standard concentration levels of Mix 155 along with Neostigmine as standard inhibitor and analyzed in order of increasing concentration. The values of RSD for 5 repetitions are found to be in a range of 1.51 – 34.45. The detection limit is found to be below 1 ppb.

The method is utilized for real cotton samples extracted with different solvents (methanol, hexane, toluene). We are able not only to estimate the inhibition % of each individual sample but also we can compare this inhibition with the standard control points. The speciality of this method is that all the samples along with the control points can be tested in one run, The total time utilized for one complete test was approximately 50 ~ 55 minutes. It is a method that offers to different investigators an easy way to detect the presence of organophosphorous and carbamate pesticides. This

method is highly sensitive, fast, simple, low-cost detection but with less Precision. Further research must be needed to verify the usefulness of the method presented here for the screening of pesticides on some more varieties of cotton of different regions. The only disadvantage is that it is only suitable for detecting AChE Inhibiting Pesticides (Organophosphorous & Carbamates). Although these pesticides share a huge portion of the total population but for the detection of other groups like Organochlorine, Pyrethroids, Benzoylureas and triazines, the development of other sensors is required.

The result of the biosensor analysis is the signal corresponding with biologic action of toxic substance. This information is more valuable in certain circumstances than the knowledge of real concentrations of pesticides. On the other side without the knowledge of the existing data correlations with actual concentrations, the data about biologic action of toxic substances are worthless. Even though biosensors do not compete with the potent chromatographic techniques, they do provide reliable analysis with a high level of sensitivity in a relatively short time. Within the prescribed optimization, the method described based on biosensors is highly useful for preliminary screening before applying more costly techniques.

In case of Algae testing, we can see that there is measurable interaction between cotton samples and algae which can be observed according to the results of our experiments. In case of Giza and Indian cotton, the organic cotton shows the stimulating effect on photosynthetic activity of the algae, where as in Pakistani cotton this is caused by the classical cotton. This is an easier, faster and cheaper method. The algal tests indicate a reasonable interaction of the analytes and the photosynthetic activity of the algae. The variation in the behavior of different cotton samples has been observed.

Clearer picture of this interaction may be observed by prolonging these tests. However algal species vary widely in their response to toxic chemicals and deferential sensitivity of green algae to the compounds has been observed in some reports. Compared with other kinds of detection devices, this method is simple and fast but it is much more sensitive for pesticide determination with much lower detection limit.

The enzymatic methods can serve as a tool for rapid, in situ screening of large numbers of samples in a short period of time but for reliable identification and quantification of compounds at ultra trace level chromatographic methods are required [137, 138].

A multiresidue method for analysis of 76 pesticides with different physicochemical properties has been developed. The pesticide residues were determined by gas chromatography with Tandem mass spectrometry (GC-MS/MS). 57 out of 76 pesticides were detected successfully by the method developed. Nineteen (19) pesticides could not be analyzed by GC-MS/MS using EI ionization, most often because of incompatibility with evaporation of the intact molecule in the GC injector. Confirmation of pesticide identity and quantitation was performed in selected-reaction monitoring mode (SRM). Trueness, Repeatability, Specificity, Limit of detection (LOD), Limit of determination (LOQ) and Applicability have been experimentally determined for each individual relevant representative analyte.

All the essential parameters which are necessary for the method validation have been taken into account in the light of the European Commission document SANCO/12495/2011 for 'Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed'[158] which is the latest version of Commission Directive 96/46/EC. The guidelines were also taken from the guidance document SANCO/825/00 'Guidance document on pesticide residue analytical methods' [159]. Moreover the document from Codex Alimentarius document 'Guidelines on Good Laboratory Practice in Pesticide Residue Analysis' has been also followed [160].

According to the guidance document SANCO/12495/2011 [158], the mean recovery should be in the range of 70–120% where as repeatability which is estimated by the relative standard deviation (RSD) of recoveries, should be ≤ 20% per commodity. According to Codex Guidelines 2003 the acceptable range of recoveries should be in between 60-120 % with a RSD value of 30 % [160].

The range of recoveries for all analytes have been varied between 81- 120 % where RSD values lied between 0.93 - 14.16 %. The accuracy and precision results for all of these analyses have been found within the acceptable range as prescribed by [158 & 160]. The LODs for KF are in the range of 0.17 - 9.84 ng/mL, for KS 0.10 - 7.87 ng/mL, for KT 0.20 – 6.69 ng/mL and for KZ it is from 0.08 – 0.83 ng/mL. Whereas the LOQs for KF are in the range of 0.56 – 32.79 ng/mL, for KS 0.33 – 26.23 ng/mL, for KT 0.67 – 23.30 ng/mL and for KZ it is from 0.27 – 2.77 ng/mL.

The method was capable of detecting pesticides in real cotton samples. The method involves a rapid and small-scale extraction procedure of real cotton samples collected

from different regions (Egypt, Pakistan & India) with five different solvents (Methanol, Acetonitrile, Acetone, Toluene, Hexane) from polar to non polar region, using Ultra Sound assisted Extraction (USE). Cryogenic Homogenization was being implemented for samples Pre-treatment. Real cotton samples extracts were analyzed in order to evaluate the applicability of the developed method. The two main strategies based on external and internal standards have been exercised for quantitative analysis of residual pesticides on cotton samples.

With External standard method, we are able to detect 26 different pesticides found in different cotton samples among which 10 pesticides are found to be exceeded than maximum residue limit (MRL) recommended by EU Pesticide Database [164] and Codex Alimentarius Commission database [165]. With Internal standard method which is a more precised way of analysis, we are able to detect 24 different pesticides which are found in all cotton samples, out of which 8 pesticides are with a value more than MRL.

The GC-MS/MS method described in this work provides a reliable procedure for the determination of residual pesticides on cotton fibers. The procedure was proven to be effective, fast, sensitive and applicable to a wide range of pesticides. In addition, using highly selective GC–MS/MS technique provided high confidence in the identification of the pesticides detected in real samples. All validation criteria mentioned by European Commission document SANCO/12495/2011 for 'Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed' [158] were fulfilled. The method gave satisfactory analytical performance parameters for the most of the targeted pesticides and analysis of real samples proved its feasibility for the intended purpose. The method described has the capability to achieve better results and efficiency for identification and quantitation of residual pesticides than the approaches currently used in pesticide residue analysis.

6.2 Recommendations for future work

Following recommendations were suggested keeping in view the results obtained through this research.

- Regarding biosensors, we calibrated the sensors with a standard pesticide mix which contains almost 18 organophosphorus pesticides. It is better to use the individual pesticide standard so that not only the identification but the quantitation can be made utilizing the biosensors as well. In this way we would also be able to see the variation between the biosensors.
- Methanol, Hexane and Toluene can be used as extracting solvent for cotton sample extraction for which we have good response with biosensors.
- In case of life cycle assessment of algae, we saw a variation in the response from different cotton samples. Also this response was more stimulating rather inhibiting. It means that the extracts from cotton samples to be used must be more concentrated to have a significant photosynthetic inhibition.
- We have used five different solvents for extraction of pesticides from cotton samples and four different standard pesticide mixes were utilized for evaluation. It is better to use one solvent and only one mix. In this way we would be able to have smaller values for limit of detection (LOD) and limit of Quantitation (LOQ).
- Other latest techniques for the extraction of residual pesticides from cotton samples like Solid phase Micro extraction (SPME), Supercritical Fluid Extraction (SFE) and Matrix Solid Phase Dispersion (MSPD) must be exercised to have more concentrated and purified extracts for better efficiency and results.

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Appendices

Appendix 1: Description of the pesticide Mix 155

Appendix 2: Description of the pesticide Mix 14

Appendix 3: Description of the pesticide Mix 3

Appendix 4: Description of the pesticide Mix 17

Appendix 5: Description of the pesticide Mix 18

Appendix 6A: Description of pesticides detected with ESTD from KS

Appendix 6B: Description of pesticides detected with ESTD from KS

Appendix 7: Description of pesticides detected with ISTD from KS

Appendix 1: Description of the pesticide Mix 155

	Name of Compounds	Use Type	Class of Pesticide	World Health Organization (WHO) Acute Hazard Rankings	U.S. EPA Acute Toxicity Rankings
MIX 155	Sulfotep	Insecticide	Organophosphorus	Ia, Extremely Hazardous	Highly Toxic
	Thiometon	Insecticide	Organophosphorus	Ib, Highly Hazardous	No Consensus Value
	Simazine	Herbicide	Triazine	U, Unlikely to be Hazardous	Slightly Toxic
	Terbumeton	Herbicide	Triazine	II, Moderately Hazardous	No Consensus Value
	Terbufos	Insecticide, Nematicide	Organophosphorus	Ia, Extremely Hazardous	No Consensus Value
	Terbuthylazine	Herbicide	Triazine	U, Unlikely to be Hazardous	Slightly Toxic
	Pirimicarb	Insecticide	N-Methyl Carbamate	II, Moderately Hazardous ©	Moderately Toxic
	Quinalphos	Insecticide	Organophosphorus	II, Moderately Hazardous	No Consensus Value
	Terbutryn	Herbicide	Triazine	U, Unlikely to be Hazardous (CP)	Slightly Toxic
	Pirimiphos-methyl	Insecticide	Organophosphorus	III, Slightly Hazardous	No Consensus Value
	Vamidothion	Insecticide	Organophosphorus	Ib, Highly Hazardous	No Consensus Value
	Tetrachlorvinphos	Insecticide	Organophosphorus	U, Unlikely to be Hazardous ©	Slightly Toxic
	Profenofos	Insecticide	Organophosphorus	II, Moderately Hazardous	No Consensus Value
	Triazophos	Insecticide	Organophosphorus	Ib, Highly Hazardous	No Consensus Value
	Triadimefon	Fungicide	Azole	III, Slightly Hazardous (CP)	Moderately Toxic
	Procymidone	Fungicide	Dicarboximide	U, Unlikely to be Hazardous ©	No Consensus Value
	Pyridiphenthion	Insecticide	Organophosphorus	III, Slightly Hazardous	No Consensus Value
	Pyrazophos	Fungicide	Organophosphorus	II, Moderately Hazardous	No Consensus Value

Appendix 2: Description of the pesticide Mix 14

	Name of Compounds	Use Type	Class of Pesticide	World Health Organization (WHO) Acute Hazard Rankings	U.S. EPA Acute Toxicity Rankings
MIX 14	Cyfluthrin	Insecticide	Pyrethroid	II, Moderately Hazardous	Moderately Toxic
	L-Cyhalothrin	Insecticide	Pyrethroid	II, Moderately Hazardous	No Consensus Value
	Cypermethrin	Insecticide	Pyrethroid	Not Listed (CP)	No Consensus Value
	Deltamethrin	Insecticide	Pyrethroid	II, Moderately Hazardous	Moderately Toxic
	Dichloran	Fungicide	Substituted Benzene	U, Unlikely to be Hazardous (CP)	Slightly Toxic
	Fenvalerate	Insecticide	Pyrethroid	II, Moderately Hazardous	Slightly Toxic
	Pendimethalin (Penoxaline)	Herbicide	2,6-Dinitroaniline	III, Slightly Hazardous (CP)	Slightly Toxic
	Permethrin	Insecticide	Pyrethroid	II, Moderately Hazardous ©	Slightly Toxic
	Tetrachlorvinphos	Insecticide	Organophosphorus	Not Listed ©	Slightly Toxic
	Tefluthrin	Insecticide	Pyrethroid	Ib, Highly Hazardous	Highly Toxic

Appendix 3: Description of the pesticide Mix 3

	Name of Compounds	Use Type	Class of Pesticide	World Health Organization (WHO) Acute Hazard Rankings	U.S. EPA Acute Toxicity Rankings
MIX 3	Captafol	Fungicide	Thiophthalimide	Ia, Extremely Hazardous	Highly Toxic
	Captan	Fungicide	Thiophthalimide	U, Unlikely to be Hazardous ©	Highly Toxic
	Demeton-S-methyl	Insecticide	Organophosphorus	Ib, Highly Hazardous	Moderately to Highly Toxic
	Demeton-S-methyl-sulfone	Insecticide	Organophosphorus	Not Listed	No Consensus Value
	Kelthane (Dicofol)	Insecticide	Organochlorine	III, Slightly Hazardous (CP)	Slightly to Highly Toxic
	Pentachlorophenol	Fungicide	Chlorinated Phenol	Ib, Highly Hazardous ©	Moderately to Highly Toxic
	Tetrachlorvinphos	Insecticide	Organophosphorus	Not Listed ©	Slightly Toxic
	Tolyfluanide	Insecticide	-----	-----	-----
	Trichlorfon (Metrifonate)	Insecticide	Organophosphorus	II, Moderately Hazardous ©	Moderately Toxic
	Vamidothion	Insecticide	Organophosphorus	Ib, Highly Hazardous	No Consensus Value

Appendix 4: Description of the pesticide Mix 17

	Name of Compounds	Use Type	Class of Pesticide	World Health Organization (WHO) Acute Hazard Rankings	U.S. EPA Acute Toxicity Rankings
MIX 17	Aldrin	Insecticide	Organochlorine	Not Listed ©	No Consensus Value
	4,4'-DDD + 2,4'-DDT	Insecticide	Organochlorine	Not Listed ©	No Consensus Value
	4,4'-DDE	Insecticide	Organochlorine	Not Listed ©	No Consensus Value
	4,4'-DDT	Insecticide	Organochlorine	II, Moderately Hazardous ©	Slightly Toxic
	Dieldrin	Insecticide	Organochlorine	Not Listed ©	No Consensus Value
	Endosulfan_I (alfa)	Insecticide	Organochlorine	Not Listed	Highly Toxic
	Endosulfan_II (beta)	Insecticide	Organochlorine	Not Listed	Highly Toxic
	Endrin	Insecticide	Organochlorine	Not Listed	Highly Toxic
	Heptachlor	Insecticide	Organochlorine	II, Moderately Hazardous©	Moderately Toxic
	Heptachlorepoxide_trans	Insecticide	Organochlorine	Not Listed	No Consensus Value
	Hexachlorobenzene	Insecticide	Organochlorine	Ia, Extremely Hazardous©	No Consensus Value
	a-HCH	Insecticide	Organochlorine	II, Moderately Hazardous©	No Consensus Value
	b-HCH	Insecticide	Organochlorine	II, Moderately Hazardous©	No Consensus Value
	g-HCH	Insecticide	Organochlorine	II, Moderately Hazardous©	No Consensus Value
	4,4'-Methoxychlor	Insecticide	Organochlorine	U, Unlikely to be Hazardous	Slightly Toxic
	Pentachlorobenzene	Fungicide	Organochlorine	Not Listed	No Consensus Value
	Pentachloronitrobenzene	Fungicide, Nematicide	Substituted Benzene	U, Unlikely to be Hazardous (CP)	Slightly Toxic
	1,2,4,5-Tetrachlorobenzene	Herbicide	Organochlorine	Not Listed	No Consensus Value
	1,2,4-Trichlorobenzene	Herbicide	Organochlorine	Not Listed	No Consensus Value

Appendix 5: Description of the pesticide Mix 18

	Name of Compounds	Use Type	Class of Pesticide	World Health Organization (WHO) Acute Hazard Rankings	U.S. EPA Acute Toxicity Rankings
MIX 18	Azinphos-methyl	Insecticide	Organophosphorus	Ib, Highly Hazardous	Highly Toxic
	Carbophenothion	Insecticide	Organophosphorus	Not Listed	No Consensus Value
	Chlorfenvinphos	Insecticide	Organophosphorus	Ib, Highly Hazardous	Highly Toxic
	Chlorpyrifos-methyl	Insecticide	Organophosphorus	U, Unlikely to be Hazardous	No Consensus Value
	Diazinon	Insecticide	Organophosphorus	II, Moderately Hazardous	Moderately Toxic
	Dichlorvos	Insecticide	Organophosphorus	Ib, Highly Hazardous ©	Highly Toxic
	Dursban	Insecticide, Nematicide	Organophosphorus	II, Moderately Hazardous	Moderately Toxic
	Dyfonate (Fonofos)	Insecticide	Organophosphorus	Ia, Extremely Hazardous	Highly Toxic
	Ethion	Insecticide	Organophosphorus	II, Moderately Hazardous	Highly Toxic
	Fenitrothion	Insecticide	Organophosphorus	II, Moderately Hazardous	Moderately Toxic
	Malathion	Insecticide	Organophosphorus	III, Slightly Hazardous (CP)	Slightly Toxic
	Methidathion	Insecticide	Organophosphorus	Ib, Highly Hazardous ©	Highly Toxic
	Methyl Parathion	Insecticide, Nematicide	Organophosphorus	Ia, Extremely Hazardous	Slightly to Highly Toxic
	Parathion	Insecticide	Organophosphorus	Ia, Extremely Hazardous (CP)	Highly Toxic
	Phosalone	Insecticide	Organophosphorus	II, Moderately Hazardous	Moderately Toxic
	Pirimiphos-methyl	Insecticide	Organophosphorus	III, Slightly Hazardous	No Consensus Value

Appendix 6A: Description of pesticides detected from KS

	Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
4,4'-DDT (0.05)	BT114_A	11053	3.094	0.012	**4,4'-DDD (0.05)**	BT114_A	19296	1.069	0.004
	BT114_T	14441	4.031	0.016		BT114_M	24401	1.351	0.005
	GC_A	8382	2.351*	0.009		GC_ACN	19207	1.064	0.004
	GC_ACN	6729	1.890*	0.008		GC_H	15679	0.868	0.003
	GC_H	7414	2.081*	0.008		GC_M	19405	1.075	0.004
	GC_M	8553	2.399*	0.010		GO_ACN	21220	1.175	0.005
	GO_A	9195	2.577*	0.010		GO_H	17950	0.994	0.004
	GO_ACN	4240	1.193*	0.005		GO_M	22213	1.230	0.005
	GO_H	9151	2.565*	0.010		IC_A	11957	0.662*	0.003
	GO_M	5292	1.488*	0.006		IC_H	17259	0.956	0.004
	IC_A	7518	2.110*	0.008		IC_M	21279	1.178	0.005
	IC_ACN	4927	1.386*	0.006		IO_A	31622	2.354	0.009
	IC_H	5638	1.585*	0.006		IO_H	41281	3.070	0.012
	IC_M	5447	1.531*	0.006		IO_M	64250	4.767	0.019
	IO_A	15649	4.365	0.017		PC_A	12008	0.665*	0.003
	IO_ACN	8384	2.352	0.009		PC_ACN	21361	1.183	0.005
	IO_H	16575	4.619	0.018		PC_M	18052	1.000	0.004
	IO_M	26535	7.339	0.029		PO_A	9366	0.519*	0.002
	PC_ACN	5324	1.497*	0.006		PO_ACN	20699	1.146	0.005
	PC_H	3978	1.1197*	0.004		PO_M	23790	1.318	0.005
	PC_M	3694	1.040*	0.004		SH1_A	17709	0.981	0.004
	PO_ACN	5112	1.438*	0.006		SH1_H	19669	1.089	0.004
	PO_H	8868	2.486*	0.010		SH1_M	25932	1.436	0.006
	PO_M	10011	2.805*	0.011		Z33_H	15958	0.884	0.004
	SH1_A	7511	2.108*	0.008		Z33_M	23100	1.279	0.005
	SH1_H	4607	1.296*	0.005	**1,2,4-Trichlorobenzene**	BT114_M	17544	1.552	0.006
	Z33_ACN	6233	1.751*	0.007		GC_A	15412	1.363	0.005
	Z33_H	5247	1.475*	0.006		GC_ACN	19956	1.765	0.007
Aldrin (0.02)	BT114_A	25235	18.409	**0.074**		GO_A	18126	1.603	0.006
	GC_A	15502	11.359	**0.045**		IC_A	19399	1.716	0.007
	GO_A	22416	16.373	**0.065**		IC_M	20223	1.789	0.007
	IO_A	19555	13.049	**0.052**		IO_A	19564	1.731	0.007
	IO_ACN	7170	4.815	0.019		IO_M	48819	4.314	0.017
	PC_A	16761	12.274	**0.049**		SH1_M	21703	1.920	0.008
	PO_A	12708	9.324	**0.037**		Z33_T	39040	3.451	0.014
	PO_ACN	6371	4.281	0.017		Z33_ACN	16188	1.432	0.006

* Values >LOD but < LOQ.

Analytes that exceed MRL are in **bold** type.

Appendix 6B: Description of pesticides detected from KS

	Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
4,4'-DDE (0.05)	BT114_A	14875	1.998	0.008	b-HCH (0.02)	BT114_A	3158	2.458	0.010
	BT114_H	12383	1.664	0.007		GC_A	1221	0.949*	0.004
	BT114_M	9924	1.333	0.005		GO_A	1324	1.025*	0.004
	GC_ACN	8265	1.110	0.004		GO_ACN	1124	0.873*	0.003
	GC_H	12135	1.630	0.007		GO_M	684	0.531*	0.002
	GC_M	10039	1.349	0.005		IC_A	1330	1.033*	0.004
	GO_ACN	10337	1.389	0.006		IO_A	1969	1.531	0.006
	GO_H	16215	2.178	0.009		IO_H	2961	2.598	0.010
	GO_M	10861	1.459	0.006		IO_M	3167	2.778	0.011
	IC_A	16015	2.152	0.009		PO_A	1803	1.401	0.006
	IC_ACN	9846	1.323	0.005		PO_H	785	0.609*	0.002
	IC_H	15308	2.057	0.008	g-HCH	BT114_A	9285	3.626	0.015
	IO_A	109675	14.746	**0.059**		BT114_M	9625	3.758	0.015
	IO_ACN	110958	14.918	0.060		GO_A	7956	3.109	0.012
	IO_H	127691	17.171	**0.069**		GO_ACN	3113	1.219*	0.005
	PC_A	11646	1.564	0.006		IC_A	5253	2.055	0.008
	PC_ACN	8014	1.077	0.004		IO_ACN	2933	0.723*	0.003
	PC_M	10027	1.347	0.005		PC_A	5185	1.278*	0.005
	PO_A	12914	1.735	0.007		PC_ACN	2912	0.718*	0.003
	PO_ACN	7030	0.944	0.004		PO_A	9775	2.410	0.010
	PO_M	12202	1.639	0.007		PO_ACN	2898	0.714*	0.003
	SH1_A	14333	1.925	0.008		Z33_T	5061	1.254	0.005
	SH1_H	11151	1.498	0.006		Z33_ACN	3020	0.744*	0.003
	SH1_M	9051	1.216	0.005	4,4'-Methoxychlor (0.01)	BT114_H	2400	2.630	**0.011**
	Z33_ACN	6064	0.815	0.003		BT114_M	2288	2.516	**0.010**
	Z33_M	10958	1.472	0.006		SH1_A	7810	7.412	**0.030**
a-HCH (0.02)	BT114_A	10309	3.713	0.015		SH1_ACN	9790	8.915	**0.036**
	BT114_T	30595	6.235	**0.025**		SH1_H	8825	8.195	**0.033**
	GO_A	15607	3.186	0.013		SH1_M	8180	7.700	**0.031**
	GO_ACN	6593	2.378	0.010		Z33_T	1034	1.188	0.005
	IC_A	10702	3.854	0.015		Z33_H	1327	1.509	0.006
	PC_A	8266	1.689*	0.007		Z33_M	7604	7.249	**0.029**
	PC_ACN	5071	1.036*	0.004					
	PO_ACN	5340	1.091*	0.004					
	Z33_T	72005	14.607	**0.058**					

* Values >LOD but < LOQ.

Analytes that exceed MRL are in **bold** type.

Appendix 7: Description of pesticides detected with ISTD from KS

	Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)		Analyte	Area Ratio	Calculated Amount (ng/mL)	Amount in samples (mg/Kg)
4,4'-DDE (0.05)	BT114_M	0.023	0.209*	0.001	**a-HCH (0.02)**	BT114_A	0.065	1.308*	0.005
	GC_ACN	0.024	0.214*	0.001		BT114_T	0.116	1.488*	0.006
	GC_M	0.020	0.178*	0.001		GO_A	0.112	1.430*	0.006
	GO_ACN	0.029	0.261*	0.001		GO_ACN	0.032	0.635*	0.003
	GO_H	0.024	0.216*	0.001		IC_A	0.070	1.409*	0.006
	GO_M	0.027	0.246*	0.001		PC_A	0.076	0.969*	0.004
	IC_A	0.026	0.236*	0.001		Z33_T	0.291	5.850	**0.023**
	IC_ACN	0.045	0.408*	0.002	**b-HCH (0.02)**	BT114_A	0.018	0.862*	0.003
	IC_H	0.030	0.267*	0.001		IO_A	0.015	0.717*	0.003
	IC_M	0.052	0.468	0.002		IO_H	0.013	0.635*	0.003
	IC_T	0.023	0.204*	0.001		IO_M	0.015	0.736*	0.003
	IO_A	0.591	5.395	0.022		PO_A	0.015	0.726*	0.003
	IO_ACN	0.692	6.337	0.025	**g-HCH**	BT114_A	0.096	1.985	0.008
	IO_H	0.611	5.587	0.022		BT114_M	0.052	1.074*	0.004
	IO_M	0.763	6.987	0.028		GO_A	0.078	1.613*	0.006
	PC_A	0.017	0.156*	0.001		IC_A	0.039	0.537*	0.002
	PC_ACN	0.018	0.159*	0.001		PC_A	0.047	0.644*	0.003
	PO_A	0.024	0.213*	0.001		PO_A	0.143	1.967	0.008
	PO_ACN	0.020	0.185*	0.001		Z33_T	0.320	4.425	0.018
	SH1_M	0.017	0.153*	0.001	**4,4'-DDD (0.05)**	BT114_A	0.082	0.297*	0.001
	Z33_ACN	0.016	0.148*	0.001		BT114_M	0.064	0.232*	0.001
	Z33_M	0.017	0.153*	0.001		GO_A	0.096	0.349*	0.001
1,2,4-Trichlorobenzene	BT114_M	0.103	1.002	0.004		GO_M	0.066	0.239*	0.001
	GC_ACN	0.067	0.662*	0.003		IC_A	0.096	0.348*	0.001
	GO_A	0.141	1.361	0.005		IO_A	0.167	0.608*	0.002
	IC_A	0.135	1.307	0.005		IO_H	0.155	0.563*	0.002
	IC_M	0.110	1.068	0.004		IO_M	0.182	0.662*	0.003
	IO_A	0.144	1.385	0.006		PC_A	0.096	0.349*	0.001
	IO_M	0.191	1.821	0.007		PO_A	0.142	0.515*	0.002
	SH1_M	0.117	1.140	0.005		PO_M	0.065	0.235*	0.001
	Z33_T	0.165	1.582	0.006		SH1_A	0.068	0.245*	0.001
	Z33_ACN	0.071	0.698*	0.003	**4,4'-Methoxychlor (0.01)**	BT114_H	0.008	0.223*	0.001
Aldrin (0.02)	BT114_A	0.285	12.099	**0.048**		BT114_M	0.033	0.921	0.004
	GC_ACN	0.068	2.857	0.011		SH1_A	0.039	1.073	0.004
	GO_A	0.280	11.921	**0.048**		SH1_ACN	0.049	1.349	0.005
	IO_A	0.260	11.045	**0.044**		SH1_H	0.042	1.166	0.005
	IO_ACN	0.076	3.183	0.013		SH1_M	0.063	1.740	0.007
	PC_A	0.298	12.671	**0.051**		Z33_T	0.013	0.361*	0.001
	PO_A	0.460	13.277	**0.053**		Z33_M	0.059	1.636	0.007
	PO_ACN	0.083	3.450	0.014					

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.